Biochemical and Biophysical Research Communications 441 (2013) 405–410

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Pigment epithelium-derived factor (PEDF) binds to caveolin-1 and inhibits the pro-inflammatory effects of caveolin-1 in endothelial cells

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ARTICLE INFO

Article history:
Received 30 September 2013
Available online 23 October 2013

Keywords:
Caveolin-1
PEDF
Inflammation
Endothelial cells
MALDI-TOF mass spectrometry

ABSTRACT

Pigment epithelium-derived factor (PEDF) exerts atheroprotective effects both in cell culture and animal models through its anti-oxidative and anti-inflammatory properties. Caveolin-1 (Cav), a major protein component of caveolae in endothelial cells (ECs), plays a role in the progression of atherosclerosis. However, effects of PEDF on Cav-exposed ECs remain unknown. In this study, we examined whether and how PEDF could inhibit the Cav-induced inflammatory and thrombogenic reactions in human umbilical vein ECs (HUVECs). Surface plasmon resonance revealed that PEDF bound to Cav at the dissociation constant of 7.36×10^{-7} M. Further, one of the major Cav-interacting proteins in human serum was identified as PEDF by peptide mass fingerprinting analysis using BIAcore 1000 combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Exogenously added Cav was taken up into the membrane fraction of HUVECs and dose-dependently increased monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1) and plasminogen activator inhibitor-1 (PAI-1) mRNA levels, all of which were blocked by the simultaneous treatment with 10 nM PEDF. Small interfering RNAs directed against Cav decreased endogenous Cav levels and suppressed gene expression of MCP-1, VCAM-1 and PAI-1 in HUVECs. This study indicates that PEDF binds to Cav and could block the inflammatory and thrombogenic reactions in Cav-exposed HUVECs. Our present study suggests that atheroprotective effects of PEDF might be partly ascribed to its Cav-interacting properties.

1. Introduction

Pigment epithelium-derived factor (PEDF) is a glycoprotein that belongs to the superfamily of serine protease inhibitors [1]. It was first purified from the conditioned media of human retinal pigment epithelial cells as a factor with potent neuronal differentiating activity [1]. Later, PEDF was found to be a highly effective inhibitor of angiogenesis both *in vitro*- and *in vivo*-models [2,3]. Furthermore, recently, we, along with others, have found that PEDF not only blocks cytokine- or growth factor-induced endothelial cell (EC) damage, platelet aggregation and T cell activation, but also prevents vascular hyperpermeability, thrombus formation, neointimal hyperplasia and cardiac remodeling in animal models through its anti-oxidative and anti-inflammatory properties [4–14]. These findings suggest that PEDF could also have atheroprotective properties [4–14].

Caveolin-1 (Cav) is a major protein component of caveolae, a specialized subset of membrane lipid raft in ECs, which are involved in multiple cellular processes such as molecular transport, cell adhesion, and signal transduction [15,16]. Indeed, Cav-1 has been shown to inhibit endothelial nitric oxide synthase activity and resultantly to impair endothelial function, which would contribute to the development of atherosclerosis [17]. Further, Cav deficiency has been reported to protect against the progression of diabetic nephropathy in mice and fatty streak lesion formation in atherosclerotic animal model [15,16,18], thus further supporting the pathological role of Cav in vascular damage and accelerated atherosclerosis. However, effects of PEDF on Cav-exposed ECs remain unknown. In this study, we examined whether and how PEDF could inhibit the Cav-induced inflammatory and thrombogenic reactions in human umbilical vein ECs (HUVECs).

2. Materials and methods

2.1. Materials

Antibodies (Abs) directed against Cav and PEDF were purchased from Cell Signaling Technology Japan K.K. (Tokyo, Japan) and R&D

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http://dx.doi.org/10.1016/j.bbrc.2013.10.074

Systems (Minneapolis, MN, USA), respectively. Abs raised against GAPDH, cadherin and calpain from Santa Cruz Biotechnology Inc. (Dallas, TX, USA).

2.2. Expression and purification of Cav

Full-length human Cav (residues 1–178) was amplified by polymerase chain reaction (PCR), sub-cloned into the *NdeI* and *XhoI* sites of the pET21b vector incorporating pentahistidine-tag (His_6-tag) into the C-terminus of the protein, and purified as described previously [19].

2.3. Preparation of PEDF proteins

PEDF proteins were purified as described previously [20]. SDS–PAGE analysis of purified PEDF proteins revealed a single band with a molecular mass of about 50 kDa, which showed positive reactivity with monoclonal Abs raised against human PEDF (Transgenic, Kumamoto, Japan).

2.4. Surface plasmon resonance (SPR)

Recombinant human His_6-tag Cav (His-rCav) was immobilized via the amino groups to CM5 sensor chip (GE Healthcare, Buckinghamshire, UK) with the aid of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and *N*-hydroxysuccinimide. For affinity measurements, the association and dissociation phases were monitored in a BIAcore 1000 (GE Healthcare). Recombinant human PEDF was injected into the flow cell at concentrations of 1.0 and 5.0 μM at a flow rate of 10 μl/min at 25 °C. The sensor chip was regenerated with pulses of 20 mM Tris–HCl buffer (pH 8.0) containing 6 M urea to the baseline level, followed by an extensive washing with the running buffer. Control experiments were performed with His-rCav-free channel on the same sensor chip. From the assay curves obtained, the control signals, reflecting the bulk effect of buffer, were subtracted using BIA-evaluation 4.1 software (GE Healthcare). Equilibrium dissociation constant (K_D) was determined using the equation for 1:1 Langmuir binding.

2.5. Isolation and identification of Cav-interacting proteins in human serum

His-rCav was immobilized as described above. Human serum (one was derived from healthy man, and the other from poorly controlled diabetic man) was diluted at 0.2 μg/ml, and 20 μl aliquots were injected over His-rCav-attached sensor surfaces. After washing, His-rCav-binding proteins were eluted with 20 mM Tris–HCl buffer (pH 8.0) containing 6 M urea or with 50 mM NaOH according to the method of BIAcore instrument. The recovered protein mixture was centrifuged at 10,000*g* for 60 min in Microcon-3 (Millipore, Bedford, MA, USA). The sample was treated with 40 μl of 1.5×Laemmli's sample buffer for 10 min at 95 °C, separated by electrophoresis using SDS–PAGE (4–12%), and then stained with silver nitrate. Silver-stained spots were excised, digested and sequenced according to the method of Wilm et al. with some modification [21]. Digested peptides were extracted with 5% (v/v) trifluoroacetic acid in 50% (v/v) acetonitrile, and subjected to peptide mass fingerprinting using a Bruker Autoflex instrument (Bruker Daltnics, Billerica, MA, USA) with an α-cyano-4-hydroxyl-*trans*-cinnamic acid matrix (Sigma, St. Louis, MO, USA). All matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were calibrated externally using a standard peptide mixture (Bruker Daltnics). Peptide mass fingerprints were searched against the national center for biotechnology information non-redundant mammalian database using ProFound search engine [22].

2.6. Cells

HUVECs were cultured in endothelial basal medium supplemented with 2% fetal bovine serum, 0.4% bovine brain extracts, 10 μg/ml human epidermal growth factor and 1 μg/ml hydrocortisone according to the supplier's instructions (Clonetics Corp., San Diego, CA). PEDF or His-rCav treatment was carried out in a medium lacking epidermal growth factor and hydrocortisone.

2.7. Preparations of membrane and cytosolic fractions of HUVECs

Membrane and cytosolic fractions of HUVECs were obtained with a ProteoExtract Subcellular Proteome Extraction kit according to the supplier's recommendation (Merck K.K. Tokyo, Japan).

2.8. Western blotting analysis

His-rCav-interacting proteins in human serum were recovered as mentioned above, while proteins were extracted from transfected or non-transfected HUVECs with lysis buffer as described previously [23]. Then the samples were separated by SDS–PAGE and transferred to nitrocellulose membranes. Membranes were probed with Abs against PEDF, His_6 (R&D Systems), Cav, cadherin, calpain, or GAPDH, and then immune complexes were visualized with an enhanced chemiluminescence detection system (Amersham Bioscience, Buckinghamshire, UK).

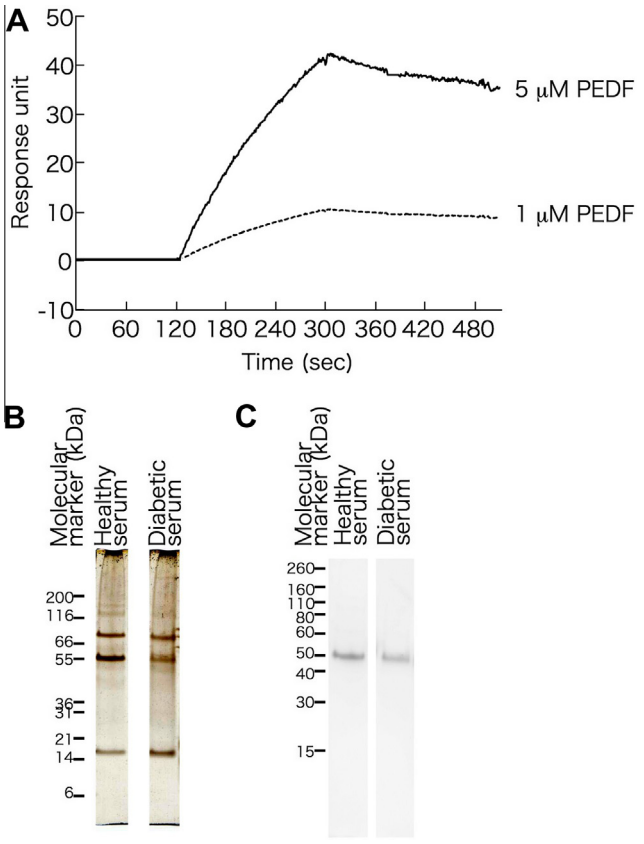

Fig. 1. Binding affinity of PEDF to His-rCav (A) and His-rCav-interacting proteins in human serum (B) and (C). (A) Representative binding sensorgram of PEDF to immobilized His-rCav. $N = 3$ per group. (B) Silver staining of eluted His-rCav-interacting proteins in human serum from healthy control and diabetic man. (C) His-rCav-interacting proteins in human serum were analyzed by Western blots using anti-PEDF Ab. Three independent experiments were performed and obtained the same results.

2.9. Construction and transfection of small interfering RNAs (siRNAs)

Sense and antisense human Cav siRNAs (siCav) used in this experiment (5′-GCUUCCUGAUUGAGAUUCAtt-3′ and 5′-UGAAUCUCAAUCAGGAAGCtc, respectively) were obtained from Life Technologies Japan Ltd. (Tokyo, Japan). Control non-silencing siRNAs (siCon) were also obtained from Life Technologies Japan Ltd. (Silencer Negative Control #1 siRNA). Then the siRNA duplexes were transfected to HUVECs using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) as described previously [24]. After 3 days of transfection, Cav and GAPDH protein levels were analyzed with western blots.

2.10. Real-time reverse transcription-PCR (RT-PCR)

Transfected or non-transfected HUVECs were treated with or without the indicated concentrations of His-rCav in the presence or absence of 10 nM PEDF for 4 h. Then total RNA was extracted with RNAqueous-4PCR kit (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed using Assay-on-Demand and TaqMan 5 fluorogenic nuclease chemistry (Applied Biosystems) according to the supplier's recommendation. IDs of primers for human monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), plasminogen activator inhibitor-1 (PAI-1), and b-actin gene were Hs00234140_m1, Hs01003372_m1, Hs01126604_m1, and Hs01060665_g1, respectively.

2.11. Statistical analysis

All values were presented as mean ± standard error. One-way ANOVA followed by the Scheffe F test was performed for statistical comparisons; $p < 0.05$ was considered significant.

3. Results

We first examined the binding affinity of PEDF to His-rCav. As shown in Fig. 1A, the representative binding sensorgram of 1 and 5 mM PEDF to immobilized Cav showed that PEDF bound to His-rCav; K_D value was $7.36 \times 10^{-7} \pm 1.18 \times 10^{-7}$ M.

We further investigated whether His-rCav-interacting proteins could exist in human serum. Clinical characteristics of 1 healthy control and 1 diabetic patient are shown in Supplementary Table 1. As shown in Fig. 1B, three major His-rCav-binding proteins at approximate molecular mass of 66-, 50-, and 14-kDa were detected in silver staining. Peptide mass fingerprinting analysis of tryptic fragments of the ca. 50-kDa protein using MALDI-TOF mass spectrometry and ProFound search engine identified the protein as PEDF; major mass peaks detected by mass spectrometry analysis, and the corresponding amino acid sequences of human PEDF, were as follows: m/z 718.389 for ^{215}GQWVTK^{220}, m/z 904.454 for ^{241}VPMMSDPK^{248}, m/z 1,025.525 for ^{319}LSYEGEVTK^{327}, m/z 1,055.643 for ^{307}TVQAVLTVPK^{316}, m/z 1,214.644 for ^{124}ELLDTVTAPQK^{134}, and m/z 1250.669 for ^{400}DTDTGALLFIGK^{411} (GenBank ID: AAA60058.1; amino acid sequence for human PEDF). Total peptide coverage corresponded to 13% of the predicted peptide sequence with a ProFound expectation score of 0.018. As shown in Fig. 1C, the 50-kDa protein was confirmed to be PEDF by Western blot analysis. There was no difference of intensity of bands between healthy control and diabetic subject. We found that the bands at 66- and 14-kDa were non-specific ones because they were identified as type II keratin and IgG heavy chain variable region, respectively.

We next studied whether exogenously added His-rCav was taken up into the membrane fraction of HUVECs. As shown in

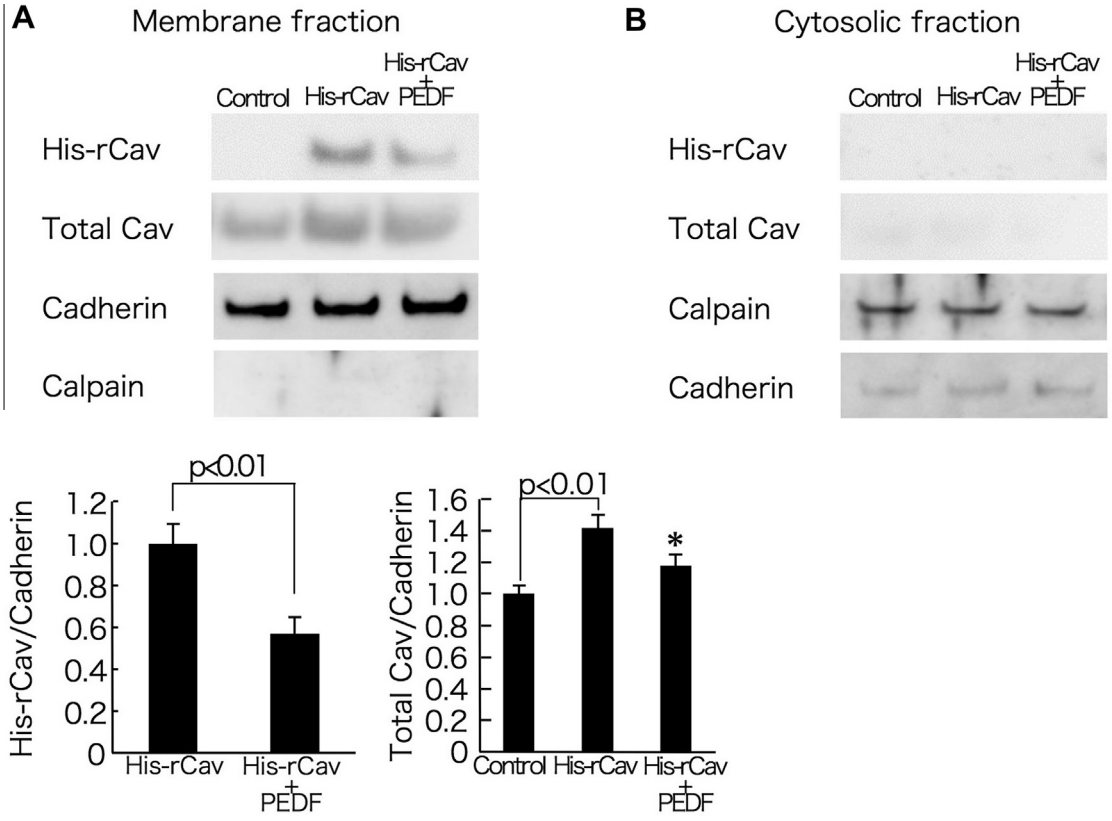

Fig. 2. Uptake of exogenously added His-rCav into the membrane (A) and cytosolic fractions (B) of HUVECs. HUVECs were treated with or without 10 nM His-rCav in the presence or absence of 10 nM PEDF for 4 h. Then membrane and cytosolic fractions of HUVECs were obtained. The samples were separated by SDS–PAGE and subjected to western blot analysis using Abs raised against His6, Cav, a membrane protein cadherin, or a cytosolic protein calpain. $N = 6$ per group. $^*p < 0.05$ compared to the value with His-rCav treatment alone.

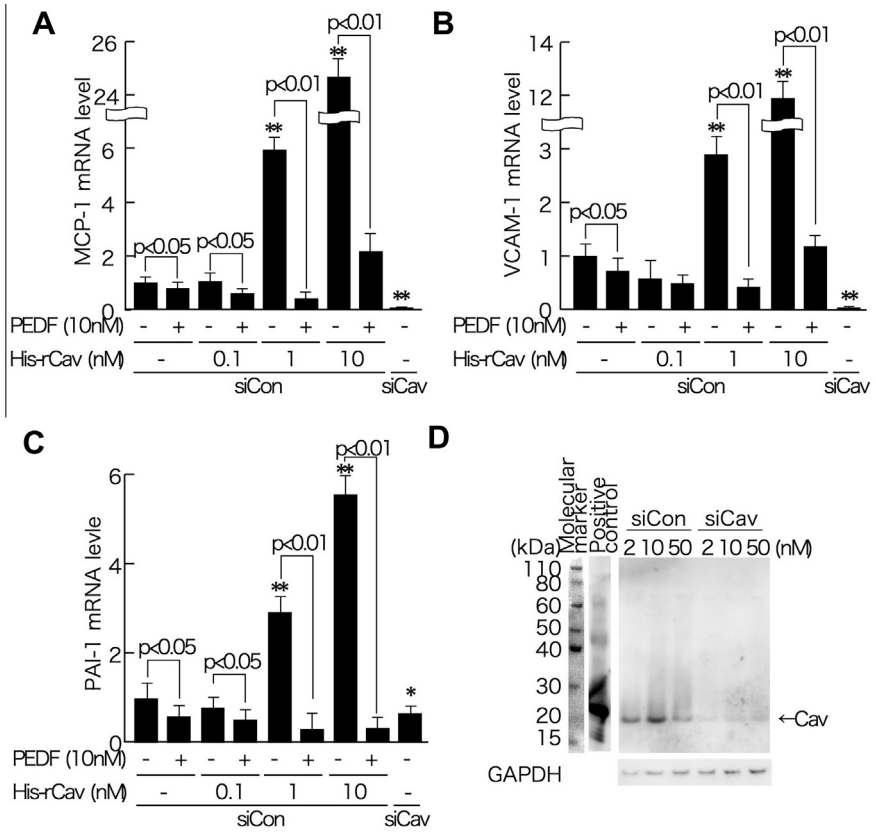

Fig. 3. Effects of PEDF on inflammatory and thrombogenic reactions in His-rCav-exposed HUVECs. siCon- or siCav-transfected HUVECs were treated with or without the indicated concentrations of His-rCav in the presence or absence of 10 nM PEDF for 4 h. (A–C) Total RNAs were transcribed and amplified by real-time PCR. Data were normalized by the intensity of β-actin mRNA-derived signals and then related to the value obtained with siCon treatment alone. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the control value, respectively. (D) Proteins were extracted from HUVECs. Western blot analysis was performed using anti-Cav Ab. $N = 3$ per group.

Fig. 2A and B, exogenously added His-rCav was actually taken up into the membrane, but not cytosolic fractions of HUVECs, which was blocked by the simultaneous treatment with 10 nM PEDF. Resultantly, His-rCav treatment increased total Cav levels in the membrane of HUVECs, which was also suppressed by PEDF (Fig. 2A).

We further examined the effects of PEDF on inflammatory and thrombogenic reactions in His-rCav-exposed HUVECs. As shown in Fig. 3A–C, exogenously added His-rCav dose-dependently increased mRNA levels of MCP-1, VCAM-1 and PAI-1 in siCon-transfected HUVECs, all of which were inhibited by the treatment with 10 nM PEDF. siCav transfection dramatically reduced endogenous Cav expression levels (Fig. 3D) and suppressed gene expression of MCP-1, VCAM-1 and PAI-1 in HUVECs (Fig. 3A–C).

4. Discussion

We have previously found that PEDF inhibits high glucose-, tumor necrosis factor-α- or angiotensin II-elicited cell damage in HUVECs through its anti-oxidative properties [4,25,26]. A couple of PEDF binding proteins have so far been reported [27,28]. Among them, laminin receptor has been shown to mediate the anti-angiogenic effects of PEDF in ECs [27]. However, little is known about how PEDF exerts its anti-oxidative and anti-inflammatory effects in HUVECs. In this study, we have demonstrated for the first time that (1) PEDF directly binds to His-rCav at K_D value of 7.36×10^{-7} M, (2) His-rCav-interacting proteins exists in PEDF in human serum, and one of the major proteins is PEDF, (3) exogenously added His-rCav is taken up into the membrane fraction of HUVECs, increases membrane levels of Cav, and dose-dependently up-regulates mRNA levels of MCP-1, VCAM-1 and PAI-1 in these cell types, (4) siCav treatment reduces Cav levels and suppresses gene expression of MCP-1, VCAM-1 and PAI-1 in HUVECs, and (5) 10 nM PEDF significantly blocks the uptake of His-rCav into the membrane faction and inhibits MCP-1, VCAM-1 and PAI-1 gene induction in His-rCav-exposed HUVECs. These observations indicate that PEDF binds to Cav and could block the inflammatory and thrombogenic reactions in Cav-exposed HUVECs.

There are several reports to show the direct involvement of endothelial Cav in the pathogenesis of endothelial cell damage and atherosclerosis [15,18,29,30]. Global loss of Cav in atherosclerosis-prone, apoE-deficiency mice has been shown to inhibit MCP-1 and VCAM-1 expression and reduce macrophage infiltration into the atherosclerotic plaques, whose effects were completely reversed by re-expression of Cav in endothelium [29]. Furthermore, endothelial-specific overexpression of Cav increases the number and size of aortic plaques and VCAM-1 expression in the vessel wall of Western-type diet-fed ApoE knockout mice [30]. These findings demonstrate that endothelial Cav could have pro-inflammatory and atherosclerosis-promoting effects in animal models. So, our present study suggests that atheroprotective properties of PEDF [9–14] might be partly ascribed to its Cav-interacting and inhibitory ability.

We, along with others, have previously reported that human blood concentration of PEDF is about 100–200 nM [31,32]. However, most of circulating PEDF in serum could exist as a protein-bound and inactive from [30]. In addition, we have previously shown that the same concentration of PEDF (10 nM) has anti-

inflammatory and anti-oxidative properties in cytokine- or growth factor-exposed HUVECs [4,25,26]. This is a reason why we chose the concentration of PEDF at 10 nM in the present experiments. Since 10 nM PEDF alone did not evoke oxidative stress generation or inflammatory reactions in HUVECs [4,25,26], it is unlikely that the present effects of PEDF on HUVECs were non-specific and toxic ones. Recently, serum levels of Cav are shown to be 0.1–1 nM, and the values are increased especially in patients with advanced stage of prostate cancer [33]. Epithelial cell hyperplasia in the prostate associated with increased angiogenesis was observed in PEDF-deficient mice [34]. Moreover, in contrast to the case of Cav [35], PEDF levels in prostate cancer tissues were dramatically decreased compared with those in benign prostatic hyperplasia tissues. These findings further support the clinical relevance of PEDF and Cav interaction in vascular damage *in vivo*.

MCP-1 plays an important role in the early phase of atherosclerosis by initiating monocyte recruitment to the vessel wall, and its expression is elevated in human atherosclerotic plaques [36,37]. The selective targeting of CCR2, the receptor for MCP-1, markedly decreases atheromatous lesion formation in apoE knockout mice [36,37]. Moreover, one early phase of atherosclerosis involves the recruitment and firm adhesion of inflammatory cells to ECs, whose process is mediated by VCAM-1 [38]. Attenuated fibrinolytic activity due to increased PAI-1 levels is prevalent in high-risk patients for cardiovascular disease, thus contributing to the increased risk of atherothrombosis in these subjects [39]. Therefore, our present observations suggest that PEDF may play a protective role against the development and progression of atherosclerosis partly via inhibition of pro-inflammatory effects of Cav.

Acknowledgments

This work was supported in part by Grants of MEXT-Supported Program for the Strategic Research Foundation at Private Universities, the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (S.Y.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.074.

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