Biochemical Pharmacology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

Proapoptotic PEDF functional peptides inhibit prostate tumor growth—A mechanistic study

Qing Gong [a,b,1], Shaopeng Qiu [c,1], Shuai Li [a,b,1], Yuedong Ma [d], Mingyuan Chen [e], Yachao Yao [a], Di Che [a], Juan Feng [a], Weibin Cai [a], Jianxing Ma [f], Xia Yang [a], Guoquan Gao [a,g,*], Zhonghan Yang [a,*]

[a] Department of Biochemistry, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, 510080, China
[b] Department of Biochemistry, Guangzhou Medical University, Guangzhou, 510082, China
[c] Department of Urology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, 510080, China
[d] Department of Cardiology, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510080, China
[e] Department of Nasopharyngeal Carcinoma, Sun Yat-sen University Cancer Center, 651 Dongfeng East Road, Guangzhou 510060, China
[f] Department of Physiology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, U.S.A.
[g] Key Laboratory of Functional Molecules from Marine Microorganisms (Sun Yat-sen University), Department of Education of Guangdong Province, China

ARTICLE INFO

Article history:
Received 14 June 2014
Received in revised form 13 September 2014
Accepted 17 September 2014
Available online xxx

Keywords:
PEDF
Epitope
Prostate carcinoma
Apoptosis
PPARγ

ABSTRACT

PEDF inhibits tumor growth via anti-angiogenic activity; however, the direct effect of PEDF on prostate carcinoma and its functional epitope as well as the underlying mechanism regulating the pathway from extracellular receptors to nuclear transcription factors has not been fully elucidated. This study investigates the ability and mechanism by which the functional PEDF peptides PEDF34 and PEDF44 suppress tumor growth. The results showed that death receptor pathway was activated by PEDF34 through up-regulation of FasL and activation of caspase-8 in both xenograft tumor tissues and PC-3 cells. FasL knockdown by siRNA or JNK-p inhibition attenuated apoptosis induced by PEDF34. NF-κB and PPARγ are crucial transcription factors for FasL expression. PEDF34 up-regulated PPARγ but did not affect NF-κB. PEDF34-induced up-regulation of FasL was abolished by siRNA-mediated PPARγ knockdown or using PPARγ inhibitor GW9662, whereas inhibition of NF-κB by the inhibitor PDTC or by siRNA had no effect. Furthermore, activation of JNK is necessary for PEDF34-induced up-regulation of FasL. PEDF34 has stronger hydropathicity and more interactions with laminin receptor than PEDF44. Blocking the laminin receptor abolished the up-regulation of FasL and PPARγ by PEDF34. Moreover, PEDF34 uses a similar mechanism to induce apoptosis in both endothelial and cancer cells. This study provides evidence that PEDF34, not PEDF44, serves as the proapoptotic epitope and exerts proapoptotic activity in both cancer and endothelial cells through activation of the extrinsic death receptor pathway. The dual anti-tumor and anti-angiogenic activities of PEDF34 suggest that it may be a promising agent for the treatment of prostate cancer.

1. Introduction

Pigment epithelium-derived factor (PEDF, also known as serpin F1) is a multifunctional protein that is involved in neuroprotection, neuronal differentiation-induction, neural stem cell self-renewal, anti-inflammatory, anti-angiogenic, and anti-cancer activities [1]. A recent study further identified PEDF as an adipocyte-derived inflammatory factor [2]. Studies of retinopathy and macular degeneration show that PEDF also plays an important role in the eye, where it suppresses angiogenesis and vascular leakage [3]. PEDF has been shown to inhibit the tumor growth and invasion of lung carcinoma [4], hepatocellular carcinoma [5], osteosarcoma [6], neuroblastoma [7], retinoblastoma [8], ovarian cancer [9], cervical cancer [10], and prostate carcinoma [11] through the blockage of neovascularization. In addition to its anti-angiogenesis activity, PEDF is capable of inducing differentiation (e.g., Rb and PC-3 cells) [12], inhibiting migration (e.g., glioma and

* Corresponding authors at: Department of Biochemistry, Zhongshan School of Medicine, Sun Yat-sen University, 74 Zhongshan 2nd Road, Guangzhou 510080, China.
E-mail addresses: gaogq@mail.sysu.edu.cn (G. Gao), yangzhh@mail.sysu.edu.cn (Z. Yang).
[1] They have contributed equally to this study.

http://dx.doi.org/10.1016/j.bcp.2014.09.012
0006-2952/

melanoma cells) [13,14], and promoting apoptosis (e.g., osteosarcoma) [15] in tumor cells.

Prostate growth is dependent on angiogenesis. Mice with a PEDF deficiency show hypervascularization as well as hyperplasia in the prostate [16]. Increased carcinoma size and angiogenesis is accompanied by decreased PEDF expression in human prostate cancer [17]. In addition, PEDF expression is decreased in metastatic prostate adenocarcinoma compared to non-metastatic disease, implying that the loss of PEDF contributes to the progression of a metastatic phenotype. Finally, exogenous PEDF suppresses prostate cancer growth [11,16,18,19]. A proposed mechanism is that PEDF affects prostate cancer through anti-angiogenesis, induction of endothelial cell apoptosis, tumor cell differentiation and apoptosis.

PEDF is known to have two functional epitopes: a 34-mer (residues 24–57, PEDF34) and a 44-mer (residues 58–101, PEDF44) [11]. In prostate cancer cells, PEDF34 induces endothelial cell apoptosis and blocks angiogenesis, whereas PEDF44 promotes neuroendocrine differentiation. Previous reports have demonstrated that PEDF and a synthetic 34-mer PEDF can induce endothelial cell apoptosis via the Fas/FasL pathway [20,21]. Recently, Ho *et al.* reported that PEDF induces apoptosis of endothelial cells through the activation of cPLA2-α, which then activates p38 MAPK, peroxisome proliferator-activated receptor gamma (PPAR-γ), p53, and caspase-3 [22,23].

We previously reported the preparation of the functional peptides PEDF34 and PEDF44 using the GST-fusion system and thrombin digestion [24]. However, the functional epitope of PEDF responsible for the proapoptotic effect on cancer cells, as well as the signaling pathways involved, have not been fully characterized. The aims of this study are to: (i) examine whether PEDF34 or PEDF44 inhibit tumor growth *in vitro* and *in vivo*; (ii) identify the proapoptotic epitope of PEDF, as well as the signaling pathways involved, in cancer cells; and (iii) determine whether the proapoptotic epitope is responsible for inducing apoptosis of tumor cells as well as endothelial cells.

2. Materials and methods

2.1. Cell culture

Human prostatic carcinoma cells (PC-3, DU-145) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were characterized by DNA fingerprinting, isozyme and cell vitality detection, and DNA sequencing to assess the presence of mutations. Cells were cultured in RPMI medium 1640 (GIBCO, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (GIBCO, Gaithersburg, MD, USA) at 37 °C in a humidified incubator at 5% CO_2. Primary human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described previously [24]. Immortalized HUVEC cell lines were acquired from Professor Deng's group at the Sun Yat-sen University Cancer Center (Guangzhou, China).

2.2. Expression and purification of recombinant PEDF and epitope peptides

PEDF cDNA encoding the full-length of the mature peptide or the epitope peptides (PEDF34 and PEDF44) was amplified from the total RNA of the human liver by reverse transcription-PCR as described previously [24,25]. The sequences of the primers are listed below. PEDF mature protein (400 AA, 45 kDa): 5′ AAT GAA TTC TGC CAG AAC CCT GCC, 3′ TCA GTC GAC TTA GGG GCC CCT GGG GTC. PEDF34 epitope peptide (34 AA, 4 kDa): 5′ AAT GGA TCCGAT CCT TTC TTC AAA, 3′ TCA GAA TTC TTA GTT GGT CGT GGG GCT. PEDF44 epitope peptide (44 AA, 5 kDa): 5′ AAT GGA TCCGTG CTC CTG TCT CCT, 3′ TCA GAA TTC TTA GGT ACC ATG GAT GTC. The PEDF PCR product was cloned into the pGEX vector (Amersham Pharmacia Biotech, USA) at the *Eco*R I and *Sal* I sites (underlined). The PEDF34 and PEDF44 PCR products were cloned into the pGEX vector at the *Bam*H I and *Eco*R I sites (underlined). The recombinant vectors were transformed into BL-21/DE3 (Novagen, Inc., Madison, WI) *Escherichia coli* cells. Expression and purification of PEDF were carried out according to the GST-fusion protein purification manual.

2.3. Molecular modeling and secondary structure annotation

The secondary structure of PEDF34, PEDF44 and LamR, as well as the localization of the alkaline amino acids Lys and Arg in their epitopes, were modeled and visualized using Cn3D and Raswin software. The circular dichroism (CD) assay was used for analyzing the conformation of PEDF34 and PEDF44 peptides in solution. A linear diagram of the human PEDF partial sequences, including PEDF34 and PEDF44, was analyzed by SAS (website: http://www.ebi.ac.uk/thornton-srv/databases/sas/). SAS results were recorded as UniProt Accession No. P36955 for PEDF. Hydropathicity of PEDF34 and PEDF44 was analyzed by ProtScale (website: http://ca.expasy.org/protscale.html).

2.4. Cell viability assay

HUVECs and prostate carcinoma cell lines (PC-3 and DU-145) were seeded in gelatin (0.1%)-coated and non-gelatin-coated 12-well plates, respectively, in triplicate and cultured to 60–70% confluence. The culture medium was then replaced with medium containing 2% FBS for HUVECs or no FBS for cell lines. Cells were treated with PEDF34 or PEDF44 at a concentration of 20, 40, 80, 160, 320, 640, 1280 nmol/L. After 3 days, viable cells were quantified by the 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide (MTT) assay (Sigma Chemical Co., St. Louis, MO).

2.5. Analysis of apoptosis by TUNEL, Hoechst 33258 and Annexin V/PI staining

Deparaffinized 5-μm tumor sections were examined for apoptosis using an ApopTag Kit (Oncor, Inc., Gaithersburg, MD, USA). After the slides were deparaffinized and rehydrated, the samples were permeabilized with Proteinase K for 20 min. After blocking with 3% H_2O_2, they were labeled with the TUNEL reaction mixture for 2 h at 37 °C in a humidified atmosphere and then visualized with diaminobenzidine and hematoxylin (Dako, Denmark A/S, Denmark).

DNA chromatin morphology of apoptotic PC-3 cells was assessed using Hoechst staining. PC-3 cells were incubated with 320 nM PEDF34 and/or other predetermined reagents for 3 days. Cells were washed by PBS and labeled with 5 μg/mL of Hoechst 33258 (Sigma, St. Louis, MO, USA) for 10 min, and the cells were examined by fluorescence microscopy. Colchicine (Sigma, St. Louis, MO, USA), which is known to induce apoptosis by disrupting microtubules and preventing their polymerization, was used as a positive control.

Apoptotic cells were stained by Annexin V/PI and were further quantitated by flow cytometry. Cells were plated at a density of 10^5 cells per well in 6-well plates, exposed to PEDF34 or PEDF44 for 3 days, and harvested for Annexin V and propidium iodide (PI) staining using the Annexin V-FITC Apoptosis Detection Kit (KeyGEN, Nanjing, China). The cells were subsequently counted by flow cytometry (Coulter, Hialeah, FL). To directly identify JNK-p involvement in PEDF34-induced apoptosis, we applied two different c-Jun N-Terminal kinase (JNK/SAP kinase) inhibitors, JNK inhibitor II (SP600125; EMD-Calbiochem, San Diego, CA) and

Please cite this article in press as: Gong Q, et al. Proapoptotic PEDF functional peptides inhibit prostate tumor growth—A mechanistic study. Biochem Pharmacol (2014), http://dx.doi.org/10.1016/j.bcp.2014.09.012