Biochemical and Biophysical Research Communications 431 (2013) 693–697

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Pigment epithelium-derived factor (PEDF) inhibits survival and proliferation of VEGF-exposed multiple myeloma cells through its anti-oxidative properties

Ritsuko Seki [a], Sho-ichi Yamagishi [b], Takanori Matsui [b], Takafumi Yoshida [c], Takuji Torimura [c], Takato Ueno [c], Michio Sata [c], Takashi Okamura [a,*]

[a] Division of Hematology and Oncology, Department of Medicine, Kurume University School of Medicine, Japan
[b] Department of Pathophysiology and Therapeutics of Diabetic Vascular Complications, Kurume University School of Medicine, Kurume, Japan
[c] Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Japan

ARTICLE INFO

Article history:
Received 12 January 2013
Available online 31 January 2013

Keywords:
Multiple myeloma
PEDF
VEGF
ROS

ABSTRACT

Vascular endothelial growth factor (VEGF) has been reported not only to induce angiogenesis within the bone marrow, but also directly stimulate the proliferation and survival of multiple myeloma cells, thus being involved in the development and progression of this second most common hematological malignancy. We, along with others, have found that pigment epithelium-derived factor (PEDF) has anti-angiogenic and anti-vasopermeability properties both in cell culture and animal models by counteracting the biological actions of VEGF. However, effects of PEDF on VEGF-exposed myeloma cells remain unknown. In this study, we examined whether and how PEDF could inhibit the VEGF-induced proliferation and survival of myeloma cells. PEDF, a glutathione peroxidase mimetic, ebselen, or an inhibitor of NADPH oxidase, diphenylene iodonium significantly inhibited the VEGF-induced reactive oxygen species (ROS) generation, increase in anti-apoptotic and growth-promoting factor, myeloid cell leukemia 1 (Mcl-1) expression, and proliferation in U266 myeloma cells. VEGF blocked apoptosis of multiple myeloma cells isolated from patients, which was prevented by PEDF. PEDF also reduced p22phox levels in VEGF-exposed U266 cells. Furthermore, overexpression of dominant-negative human Rac-1 mutant mimicked the effects of PEDF on ROS generation and Mcl-1 expression in U266 cells. Our present study suggests that PEDF could block the VEGF-induced proliferation and survival of multiple myeloma U266 cells through its anti-oxidative properties via suppression of p22phox, one of the membrane components of NADPH oxidase. Suppression of VEGF signaling by PEDF may be a novel therapeutic target for multiple myeloma.

1. Introduction

Multiple myeloma is a clonal B-cell malignancy characterized by accumulation of malignant plasma cells within the bone marrow, which is clinically associated with the presence of monoclonal paraprotein in serum or urine, osteolytic lesions and renal dysfunction [1,2]. Although remarkable therapeutic advances in the treatment of multiple myeloma have been made with high-dose chemotherapy and autologous stem-cell transplantation, these therapeutic options may be far from satisfactory and limited by considerable side effects [1,2]. Therefore, to identify novel therapeutic target for multiple myeloma is urgently desired.

Various growth factors, including vascular endothelial growth factor (VEGF), interleukin-6 and insulin-like growth factor have been shown to play a role in the development and progression of multiple myeloma [1,3]. Among them, in addition to promoting angiogenesis within the bone marrow, VEGF has been reported to directly stimulate proliferation and survival of multiple myeloma cells [3–5].

Pigment epithelium-derived factor (PEDF), a glycoprotein that belongs to the superfamily of serine protease inhibitors, was first purified from the conditioned media of human retinal pigment epithelial cells as a factor which possesses potent neuronal differentiating activity [6]. PEDF has been shown to be a highly effective inhibitor of angiogenesis in cell culture and animal models; it inhibited retinal endothelial cell (EC) growth and migration and suppressed ischemia-induced retinal neovascularization [7,8]. Furthermore, recently, we, along with others, have found that PEDF not only reduces advanced glycation end product-, angiotensin II- or leptin-induced vascular endothelial growth factor (VEGF) expression, but also inhibits the biological actions of VEGF, thus exerting anti-angiogenic and anti-permeability effects both *in vitro* and *in vivo* [9–17]. However, effects of PEDF on VEGF-exposed multiple

* Corresponding author. Address: Division of Hematology and Oncology, Department of Medicine, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan. Fax: +81 942 31 7854.
E-mail address: okamura@med.kurume-u.ac.jp (T. Okamura).

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http://dx.doi.org/10.1016/j.bbrc.2013.01.057

myeloma cells remain unknown. Therefore, this study investigated whether and how PEDF could block the VEGF-induced proliferation and survival of multiple myeloma cells.

2. Materials and methods

2.1. Materials

Ebselen (2-phenyl-1, 2-benzisoselenazol-3(2H)-one), a glutathione peroxidase mimetic, was purchased from Aventis Pharma AG (Frankfurt, Germany). An inhibitor of NADPH oxidase, diphenylene iodonium (DPI) from Sigma (St. Louis, MO, USA). VEGF was obtained from PeproTech (Rocky Hill, NJ, USA). Antibodies (Abs) directed against p22phox, gp91phox, myeloid cell leukemia 1 (Mcl-1), β-actin and GAPDH from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.2. Preparation of PEDF proteins

PEDF proteins were purified as described previously [18]. SDS–PAGE analysis of purified PEDF proteins revealed a single band with a molecular weight of about 50 kDa, which showed positive reactivity with monoclonal Abs raised against human PEDF (Transgenic, Kumamoto, Japan).

2.3. Preparation of neutralizing Abs directed against PEDF

Neutralizing Abs raised against PEDF were prepared as described previously [19].

2.4. ***Cells***

Human multiple myeloma cells, U266 (ATCC, Rockville, MD, USA) and RPMI8226 (Human Science, Osaka, Japan) were cultured in RPMI1640 medium with 2 mM L-glutamine (Gibco, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) containing 100 U/ml penicillin, and 100 μg/ml streptomycin (Mediatech Inc., AK, USA). VEGF and/or PEDF treatments were carried out after cells were serum-starved overnight.

2.5. Measurement of intracellular reactive oxygen species (ROS) generation

U266 or RPMI8226 cells were treated with or without 50 ng/ml VEGF in the presence of absence of the indicated concentrations of PEDF, 5 μg/ml PEDF-Ab, 50 μM ebselen or 100 nM DPI for 24 h. Intracellular ROS generation was measured by using the fluorescent probe CM-H_2DCFDA (Molecular Probes Inc., Eugene, OR, USA) as described previously [20].

2.6. Transfection of a dominant-negative human Rac-1 mutant (DN-RacT17N)

DN-RacT17 N expression vector was kindly provided by Dr. Horiguchi, Department of Bacterial Toxinology, Research Institute for Microbial Diseases, Osaka University [21]. U266 cells were transiently transfected with either a DN-RacT17N or an empty vector with Cell Line Optimization Nucleofector Kit (Amaxa Biosystems, Cologne, Germany) as described previously [20].

2.7. Western blotting analysis

U266 cells were treated with or without 50 ng/ml VEGF in the presence of absence of 100 nM PEDF for 12 h. Then proteins were extracted from U266 cells with lysis buffer as described previously [22]. Then the samples were separated by SDS–PAGE and transferred to nitrocellulose membranes. Membranes were probed with Abs against p22phox, gp91phox, Mcl-1, or β-actin, and then immune complexes were visualized with an enhanced chemiluminescence detection system (Amersham Bioscience, Buckinghamshire, United Kingdom).

2.8. Measurement of apoptosis

Apoptotic cells were measured by flow cytometry as described previously [19]. In brief, after obtaining informed consent, mononuclear cells were isolated from bone marrow samples of multiple myeloma patients by Ficoll–Paque centrifugation (Pharmacia Biotech, Uppsala, Sweden). Plasma cells were purified from mononuclear cells with a CD138 magnetic column (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), and cultured in RPMI 1640 medium containing 0.5% FBS for 24 h. Then plasma cells were treated with or without 50 ng/ml VEGF in the presence or absence of 100 nM PEDF. After 24 h, the cells were incubated with phycoerythrin-conjugated anti-CD138 monoclonal Abs (Beckman Coulter, Hialeah, FL, USA) and fluorescein isothiocyanate-conjugated annexin V monoclonal Abs (Beckman Coulter). Both CD138- and annexin V-positive cells were quantified with a FACSort flow cytometer (BD Biosciences, Bedford, MA, USA) using a CellQuest software program according to the manufacturer's recommendation (BD Immunocytometry Systems, San Diego, CA, USA).

2.9. Cell proliferation assay

Cell proliferation was assayed by measuring 5-bromo-2′-deoxyuridine (BrdU) incorporation into newly synthesized DNA of actively proliferating cells with Cell Proliferation ELISA, BrdU colorimetric kit according to the supplier's recommendations (Roche Applied Science, Indianapolis, IN, USA). In brief, U266 cells were treated with or without 50 ng/ml VEGF in the presence of absence of the indicated concentrations of PEDF, 50 μM ebselen or 100 nM DPI for 22 h, and then BrdU was added to the medium. After 2 h-incubation, cell proliferation was quantified by measuring the absorbance at 450 nM and shown as a relative value to control.

2.10. 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 effects of PEDF on ROS generation in VEGF-exposed multiple myeloma cells. As shown in Fig. 1A, 100 nM PEDF, 50 μM ebselen or 100 nM DPI significantly inhibited the VEGF-induced increase in ROS generation in U266 cells. Furthermore, the anti-oxidative effects of PEDF were completely blocked by the simultaneous treatment with neutralizing Abs raised against PEDF. PEDF alone did not affect the ROS generation in VEGF-exposed U266 cells. Although similar results were obtained in other multiple myeloma cells, RPMI8226 cells, the effects of PEDF on VEGF-induced ROS generation were modest, not significant (Fig. 1B). Therefore, we chose U266 cells for the following experiments.

We next investigated the role of Rac-1 in VEGF-induced ROS generation in U266 cells. As shown in Fig. 1C, overexpression of DN-RacT17N significantly inhibited the VEGF-induced ROS generation in U266 cells. There were no additional ROS-reducing effects of PEDF in DN-RacT17N-transfected, VEGF-exposed U266 cells.

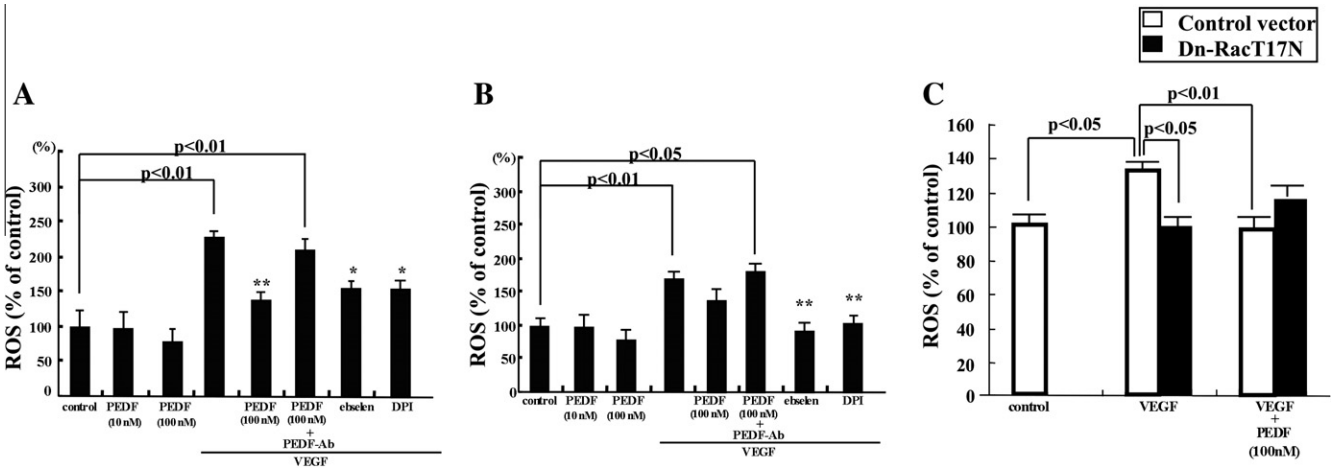

Fig. 1. Effects of PEDF on ROS generation in U266 ((A) and (C)) and RPMI8226 (B) cells. Transfected or non-transfected cells were treated with or without 50 ng/ml VEGF in the presence of absence of the indicated concentrations of PEDF, 5 μg/ml PEDF-Ab, 50 μM ebselen or 100 nM DPI for 24 h. Then intracellular ROS generation was measured by using the fluorescent probe CM-H_2DCFDA. ∗ and ∗∗, $p < 0.05$ and $p < 0.01$ compared to the value with VEGF alone, respectively. $N = 3$ per group.

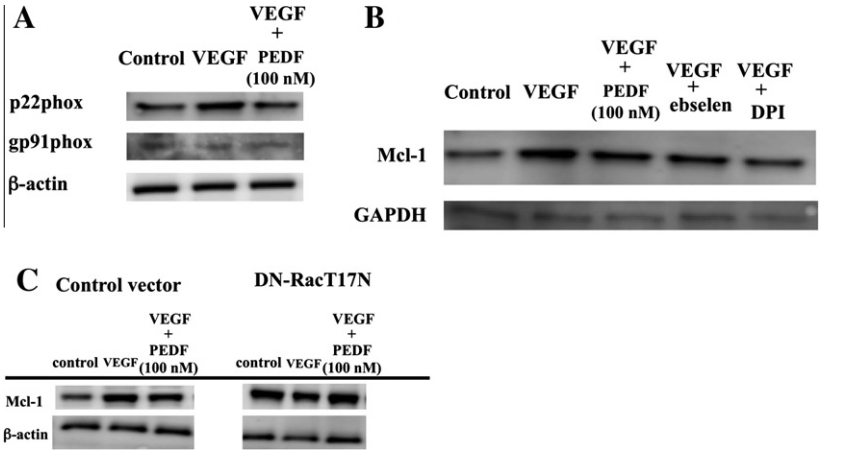

Fig. 2. Effects of PEDF on p22phox and gp91phox (A) and Mcl-1 expression levels ((B) and (C)) in U266 cells. (A) Cells were treated with or without 50 ng/ml VEGF in the presence of absence of 100 nM PEDF for 12 h. Then p22phox and gp91phox protein expression levels were evaluated by Western blot analysis. Representative bands of Western blot are shown. Three independent experiments obtained the same results. (B) and (C) Transfected or non-transfected cells were treated with or without 50 ng/ml VEGF in the presence of absence of 100 nM PEDF, 50 μM ebselen or 100 nM DPI for 12 h. Then Mcl-1 protein expression levels were evaluated by Western blot analysis. Representative bands of Western blot are shown. Three independent experiments obtained the same results. (C) Experiments in control vector-transfected cells and DN-RacT17N-transfected cells were separately performed. So, loading controls were not from the same immunoblots.

We studied the effect of PEDF on expression levels of p22phox and gp91phox, membrane components of NADPH oxidase [23]. VEGF significantly increased p22phox levels in U266 cells, which were prevented with the treatment with PEDF (Fig. 2A). Expression of gp91phox levels remained unchanged by the treatment with VEGF or PEDF.

As shown in Fig. 2B, 100 nM PEDF, 50 μM ebselen or 100 nM DPI significantly inhibited the VEGF-induced up-regulation of Mcl-1, anti-apoptotic molecule of the Bcl-2 family [24] in U266 cells. Overexpression of DN-RacT17N also blocked the Mcl-1 induction in VEGF-expose U266 cells (Fig. 2C).

Clinical characteristics of 2 multiple myeloma patients are shown in Table 1 (Supplementary material). As shown in Fig. 3, VEGF inhibited apoptosis of isolated myeloma cells, which was significantly prevented by the treatment with PEDF.

We further studied the effects of PEDF on proliferation of U266 cells. As shown in Fig. 4, and 100 nM PEDF, 50 μM ebselen or 100 nM DPI significantly suppressed the VEGF-induced

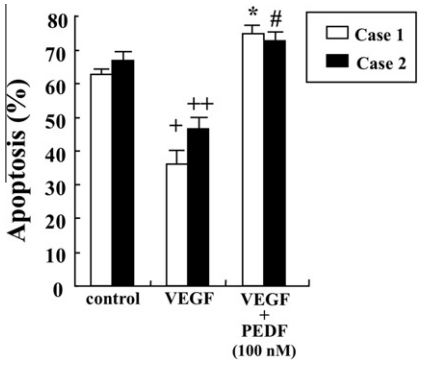

Fig. 3. Effects of PEDF on apoptosis of multiple myeloma cells isolated from patients. Isolated myeloma cells were treated with or without 50 ng/ml VEGF in the presence of absence of 100 nM PEDF for 24 h. Apoptotic cells were measured by flow cytometry. + and ++, $p < 0.05$ compared to the value with each control group. ∗ and #, $p < 0.05$ compared to the value with each VEGF alone group. $N = 4$ per group.

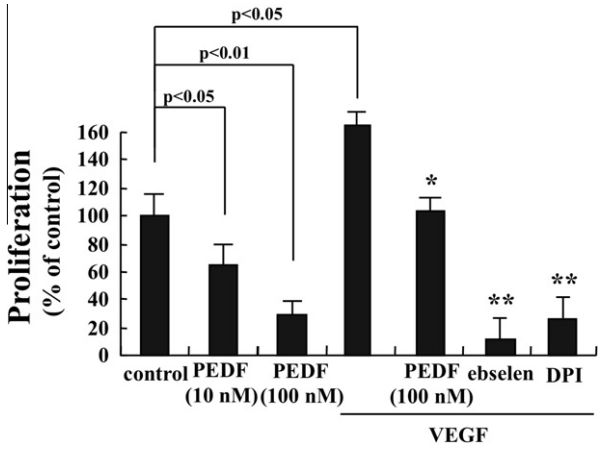

Fig. 4. Effects of PEDF on proliferation of U266 cells. Cells were treated with or without 50 ng/ml VEGF in the presence of absence of 100 nM PEDF, 50 μM ebselen or 100 nM DPI for 24 h. Cell proliferation was measured using Cell Proliferation ELISA BrdU colorimetric kit. ∗ and ∗∗, $p < 0.05$ and $p < 0.01$ compared to the value with VEGF alone, respectively. $N = 3$ per group.

proliferation of U266 cells. PEDF alone inhibited the proliferation of U266 cells in a dose-dependent manner (Fig. 4).

4. Discussion

In this study, we have demonstrated for the first time that PEDF could block the VEGF-induced proliferation and survival of multiple myeloma U266 cells through its anti-oxidative properties via suppression of p22phox, one of the membrane components of NADPH oxidase on the basis of the following evidence: (1) PEDF, a glutathione peroxidase mimetic, ebselen, an inhibitor of NADPH oxidase, DPI or overexpression of DN-RacT17N significantly inhibited the VEGF-elicited ROS generation in U266 cells; (2) PEDF significantly blocked the VEGF-induced up-regulation of p22phox in U266 cells; (3) PEDF, ebselen, DPI, or overexpression of DN-RacT17N suppressed the Mcl-1 induction by VEGF in U266 cells; (4) VEGF reduced apoptotic cell death of multiple myeloma cells isolated from patients, which were prevented by the treatment with PEDF; and (5) PEDF, ebselen, or DPI significantly inhibited the proliferation of VEGF-exposed U266 cells.

Several lines of evidence implicate VEGF as one of the key factors involved in multiple myeloma growth and metastasis [1,3–5,24]. Serum levels of VEGF have been shown to increase in patients with multiple myeloma, which is correlated with disease progression and poor prognosis of this devastating disorder [4,24]. Furthermore, VEGF is overexpressed in multiple myeloma cells, which could contribute to angiogenesis within the bone marrow and subsequently stimulate the growth, migration and survival of myeloma cells [4,24]. NADPH oxidase comprises a membrane spanning p22phox and gp91phox and 2 or more cytosolic subunits including GTPase Rac-1 [9,23]. It is well known that a number of pathophysiological stimuli induce tumor cell growth and activation as a function of NADPH oxidase-mediated ROS-induced signal transduction [23,25–27]. Moreover, NADPH oxidase-derived ROS generation is reported to be required for the angiogenic signaling of VEGF as well [23,28,29]. In this study, we found that PEDF reduced expression levels of p22phox, one of the key components of NADPH oxidase with respect to its enzymatic activity [23] and that an anti-oxidant ebselen, DPI or overexpression of DN-RacT17N mimicked the effects of PEDF on ROS generation and proliferation in U266 cells. These findings suggest that suppression of NADPH oxidase-driven ROS generation is a molecular target for anti-tumor effects of PEDF on multiple myeloma cells.

Mcl-1 belongs to the member of anti-apoptotic Bcl-2 family, and VEGF has been shown to up-regulate Mcl-1 and protect multiple myeloma cells against apoptosis [24]. Deletion of Mcl-1 is reported to reduce VEGF-induced proliferation of multiple myeloma cells as well [24]. In the present study, PEDF, ebselen, DPI or overexpression of DN-RacT17N significantly suppressed the VEGF-induced Mcl-1 expression in U266 cells. Therefore, PEDF might inhibit myeloma growth and survival by blocking the VEGF signaling via reduction of Mcl-1 levels.

The data in the present study are highly dependent on the purity of PEDF proteins prepared. However, it is unlikely that the effects of PEDF on myeloma cells were non-specific because we demonstrated here that polyclonal Abs directed against PEDF neutralized the anti-tumor effects of PEDF. We should also discuss the possibilities that the concentrations of PEDF used here were supraphysiologic levels and that PEDF may have exerted toxic effects on myeloma cells. In this regard, PEDF alone did not affect ROS generation in U266 or RPMI8226 myeloma cells. Furthermore, we, along with others, have previously reported that the human blood concentration of PEDF is about 100–200 nM [30,31]. These observations indicate that the concentrations of PEDF used *in vitro*-experiments were within a physiological range. It might be interesting to investigate whether serum levels of PEDF are decreased in multiple myeloma patients, which could be a prognostic marker of this malignancy.

Acknowledgments

This work was supported in part by Grants of Collaboration with Venture Companies Project from the Ministry of Education, Culture, Sports, Science and Technology, 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.2013.01.057.

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