Pigment Epithelium-Derived Factor (PEDF) Blocks Angiotensin II-Induced T Cell Proliferation by Suppressing Autocrine Production of Interleukin-2

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Abstract: Angiotensin II (Ang II) elicits numerous inflammatory-proliferative responses in vascular cells, thereby being involved in atherosclerosis. We have previously shown that pigment epithelium-derived factor (PEDF) blocks the Ang II-induced endothelial cell activation, thus suggesting that PEDF may play a role in atherosclerosis. However, effects of PEDF on T cell activation, another key steps of atherosclerosis, remain to be elucidated. In this study, we examined whether PEDF could inhibit the Ang II-induced MOLT-3 T cell proliferation *in vitro* and the way that it might achieve this effect. Ang II significantly stimulated DNA synthesis in MOLT-3 T cells, which was inhibited by PEDF, olmesartan, an Ang II type 1 receptor blocker, an anti-oxidant *N*-acetylcysteine (NAC), or antibodies directed against IL-2. PEDF or NAC suppressed gene expression of interleukin-2 (IL-2) in Ang II-exposed MOLT-3 T cells. Furthermore, PEDF blocked the Ang II-induced reactive oxygen species (ROS) generation and NADPH oxidase activity in MOLT-3 T cells. These results demonstrate that PEDF inhibits the Ang II-induced T cell proliferation by blocking autocrine production of IL-2 *via* suppression of NADPH oxidase-mediated ROS generation. Blockade by PEDF of T cell activation may become a novel therapeutic target for atherosclerosis.

Key Words: Atherosclerosis, angiotensin II, oxidative stress, PEDF, T cells.

INTRODUCTION

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 in human retinoblastoma cells [1]. Recently, PEDF has been shown to be a highly effective inhibitor of angiogenesis in cell culture and animal models [2]. Indeed, PEDF inhibited retinal endothelial cell (EC) growth and migration and suppressed ischemia-induced retinal neovascularization, thus suggesting that loss of PEDF in the eye is functionally important in the pathogenesis of proliferative diabetic retinopathy [3-5]. In addition, we have recently found that PEDF blocks cytokine- or growth factor-induced EC activation *via* its anti-oxidative properties [6,7]. These observations suggest that PEDF may also play a protective role against the development and progression of atherosclerosis. However, effects of PEDF on T cell growth and activation, another key steps of atherosclerosis [8], remain to be elucidated.

The renin-angiotensin system has been implicated in atherosclerosis as well [9,10]. The traditional role of the renin-angiotensin system in the context of blood pressure regulation has been modified to incorporate the concept that angiotensin II (Ang II) is a potent pro-inflammatory agent [9,10]. Indeed, Ang II stimulates EC adhesion molecule expression, elicits T cell activation, and promotes growth and migration of vascular smooth muscle cells, thus playing an important role in the development and progression of atherosclerosis [11-16]. Furthermore, recent clinical studies have demonstrated that chronic inhibition of Ang II formation or action by angiotensin-converting enzyme inhibitors or Ang II type 1 receptor antagonists, respectively, reduces the risk of cardiovascular events in patients with atherosclerosis and after myocardial infarction [17-19].

In this study, we focused on the effects of PEDF on cultured T cells. We examined here whether PEDF could inhibit the Ang II-induced MOLT-3 T cell proliferation *in vitro* and the way that it might achieve this effect.

MATERIALS AND METHODS

Materials

Ang II, 2-mercaptoethanol, *N*-acetylcysteine (NAC), diphenylene iodonium (DPI), lucigenin, and NADPH were purchased from Sigma (St. Louis, Mo.). Olmesartan, an Ang II type 1 receptor blocker was generously provided by Sankyo Co. Ltd. (Tokyo, Japan). [^{3}H]thymidine from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). Anti-human interleukin-2 (IL-2) antibody (Ab) was purchased

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1573-4064/06 $50.00+.00

from R&D systems (Minneapolis, Minn.). GeneAmp RNA PCR Core Kit was from Applied Biosystems (Branchburg, N.J.). Protease inhibitor cocktails were from Nakalai Tesque (Kyoto, Japan).

Purification 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 weight of about 50 kDa, which showed positive reactivity with monoclonal Ab against human PEDF (Transgenic, Kumamoto, Japan) [20].

Cells

MOLT-3 T cells were obtained from Health Science Research Resources Bank (Osaka, Japan) and maintained in RPMI 1640 medium (Gibco BRL, Rockville, M.D.) supplemented with 10% of fetal bovine serum (FBS) and 50 μM 2-mercaptoethanol. Ang II treatments were carried out in a medium containing 0.1% FBS.

Measurements of [^{3}H]Thymidine Incorporation

MOLT-3 T cells were treated with or without 100 nM Ang II in the presence or absence of the indicated concentration of PEDF, 100 nM olmesartan, 10 mM NAC or various concentrations of anti-IL-2 Ab for 26 h. For the last 4 h of the culture, cells were pulsed with 1 μCi [^{3}H]thymidine. Then the cells were harvested on glass-fiber filters and the radioactivity of each filter was counted.

Preparation of Polyclonal Abs against Human PEDF

Polyclonal Abs against human PEDF were prepared as described previously (Sawady Technology, Tokyo, Japan) [20]. As described in our previous paper [20], we confirmed that the newly generated polyclonal Abs actually bound to purified PEDF proteins by western blot analysis and that the Abs also completely neutralized the growth-promoting effects of PEDF on advanced glycation end product (AGE)-exposed pericytes. However, the monoclonal Abs purchased from Transgenic (Kumamoto, Japan) did not have such a neutralizing activity in AGE-exposed pericytes. The epitope of PEDF recognized by the monoclonal Abs may not be involved in biological action of this factor. These are reasons why we newly prepared polyclonal Abs although the monoclonal Abs were already available.

Primers

Primer sequences used in semi-quantitative reverse transcription-polymerase chain reactions (RT-PCR) for detecting human IL-2 mRNA were 5'-ATGTACAGGATGC AACTCCTGTCTT-3' and 5'-GTTAGTGTTGAGATGATG CTTTGAC-3' [21]. Sequences of the upstream and down stream primers used in semi-quantitative RT-PCR for detecting human β-actin mRNAs were the same as described previously [22].

Semi-Quantitative RT-PCR

Poly(A)$^+$RNAs were isolated from cells treated with or without 100 nM Ang II in the presence or absence of the indicated concentration of PEDF, 5 μg/ml polyclonal Abs against PEDF, or 10 mM NAC for 6 h, and then analyzed by RT-PCR as described previously [23]. The amounts of poly(A)$^+$RNA templates (30 ng) and cycle numbers (35 cycles for IL-2; 22 cycles for β-actin) for amplification were chosen in quantitative ranges, where reactions proceeded linearly, which had been determined by plotting signal intensities as functions of the template amounts and cycle numbers [24].

Intracellular Reactive Oxygen Species (ROS) Generation

MOLT-3 T cells were treated with or without 100 nM Ang II in the presence or absence of 100 nM PEDF, 100 nM olmesartan, or 50 nM DPI, an inhibitor of NADPH oxidase for the indicated time periods. Then intracellular ROS generation was detected by using the fluorescent probe CM-H_2DCFDA (Molecular Probes Inc., Eugene, Ore.) as described previously [25,26]. As to the specificity of measurement of ROS generation using this method, we confirmed that H_2O_2 increased the fluorescent intensity in MOLT-3 T cells in a dose-dependent manner within our assay ranges. The reproducibility of the assay was proven in three-independent experiments.

NADPH Oxidase Activity

MOLT-3 T cells were treated with or without 100 nM Ang II in the presence or absence of 100 nM PEDF for 24 h, and then the cells were suspended in homogenization buffer (20 mM Hepes, pH 7.0, 100 mM KCl, and 1 mM EDTA containing protease inhibitor cocktails). NADPH oxidase activity of the cell homogenate was measured by lumine-scence assay in 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin as the electron acceptor, and 100 μM NADPH as a substrate according to the methods of Griendling *et al.* [27].

Statistics

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

RESULTS

Effects of Various Agents on [^{3}H]Thymidine Incorporation in MOLT-3 T Cells

We first investigated effects of PEDF on [^{3}H]thymidine incorporation in Ang II-exposed MOLT-3 T cells. As shown in Fig. **1a**, **b**, Ang II stimulated DNA synthesis in MOLT-3 T cells, which was significantly blocked by PEDF, olmesartan, an Ang II type 1 receptor blocker, or an anti-oxidant NAC. Furthermore, anti-IL-2 Ab was found to inhibit the Ang II-induced DNA synthesis in a dose-dependent manner; at 10 μg/ml anti-IL-2 Ab, the growth-promoting effects of Ang II on MOLT-3 T cells were completely blocked (Fig. **1c**). PEDF, NAC, olmesartan or anti-IL-2 Ab alone did not affect DNA synthesis in MOLT-3 T cells (Fig. **1**). We confirmed that MOLT-3 T cells actually expressed IL-2 receptor (data not shown).

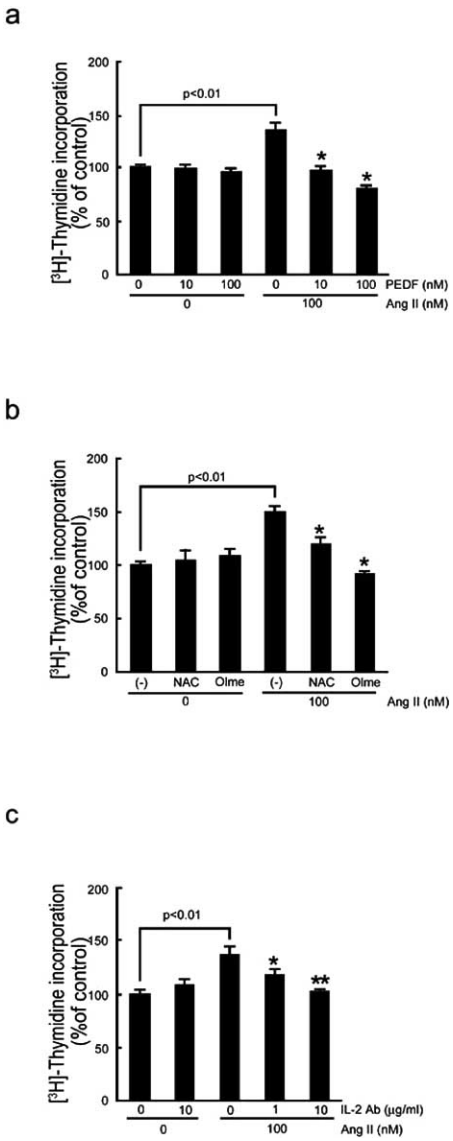

Fig. (1). Effects of various agents on [^{3}H]thymidine incorporation in Ang II-exposed MOLT-3 T cells. MOLT-3 T cells were treated with or without 100 nM Ang II in the presence or absence of the indicated concentration of PEDF (a), 10 mM NAC (b), 100 nM olmesartan (Olme) (b), or various concentrations of anti-IL-2 Ab (c) for 26 h, and then [^{3}H]thymidine incorporation was measured. The percentage of [^{3}H]thymidine incorporation is related to the value of the control. *, P <0.05; **, P <0.01 compared to the value with Ang II alone. Similar results were obtained in three independent experiments.

Effects of PEDF on IL-2 mRNA Levels in MOLT-3 T Cells

We next studied effects of PEDF on IL-2 gene expression in MOLT-3 T cells. As shown in Fig. **2a**, **b**, PEDF or NAC completely blocked the Ang II-induced up-regulation of IL-2 mRNA levels in MOLT-3 T cells. Polyclonal Abs directed against PEDF significantly reversed the effects of PEDF on IL-2 mRNA levels in Ang II-exposed MOLT-3 T cells; in the presence of PEDF Abs, Ang II increased IL-2 mRNA levels by about 1.5 fold in PEDF-exposed MOLT-3 T cells (data not shown). PEDF or NAC alone did not affect IL-2 gene expression in MOLT-3 T cells. We also confirmed that PEDF did not affect Ang II type 1 receptor mRNA levels in Ang II-exposed MOLT-3 T cells (data not shown).

Effects of PEDF on ROS Generation in MOLT-3 T Cells

We further examined whether PEDF could suppress ROS generation in Ang II-exposed MOLT-3 T cells. As shown in Fig. **3**, PEDF, olmesartan, or an NADPH oxidase inhibitor, DPI completely blocked the Ang II-induced ROS generation in MOLT-3 T cells. PEDF, olmesartan, or DPI alone did not affect ROS generationin MOLT-3 T cells. These observations suggest that Ang II-type 1 receptor interaction in MOLT-3 T cells elicited ROS generation *via* NADPH oxidase activity, which could be one of the molecular targets for PEDF as the case in ECs [7].

Effects of PEDF on NADPH oxidase activity in MOLT-3 T Cells

We next examined whether PEDF could actually suppress NADPH oxidase activity in Ang II-exposed MOLT-3 T cells. As shown in Fig. **4**, 100 nM PEDF was found to block the Ang II-induced NADPH oxidase activation in MOLT-3 T cells. However, PEDF did not affect mRNA levels of p22phox and gp91phox, two membrane components of NADPH oxidase, in Ang II-exposed MOLT-3 T cells (data not shown).

DISCUSSION

In the present study, we demonstrated for the first time that PEDF inhibited the Ang II-induced cultured T cell proliferation by blocking autocrine production of IL-2, at least in part, through its anti-oxidative properties on the basis of the following evidence: [1] Ang II significantly stimulated MOLT-3 T cell proliferation, which was completely blocked by PEDF or anti-IL-2 Ab; [2] Ang II up-regulated IL-2 mRNA levels in MOLT-3 T cells, which were also completely blocked by PEDF; [3] PEDF significantly inhibited the Ang II–elicited ROS generation in MOLT-3 T cells; [4] PEDF inhibited the Ang II-induced NADPH oxidase activity in MOLT-3 T cells; and [5] An anti-oxidant NAC mimicked the effects of PEDF on Ang II-exposed MOLT-3 T cells; NAC inhibited the Ang II-induced IL-2 gene up-regulation and subsequent increase in DNA synthesis. Since PEDF did not affect expression levels of Ang II type 1 receptor gene in Ang II-exposed MOLT-3 T cells, it was unlikely that PEDF exerted the above-mentioned effects by blocking the direct interaction of Ang II with type 1 receptor. Taken together, our present data suggests that NADPH oxidase-mediated ROS generation is a molecular target for PEDF in MOLT-3 T cells.

The data presented here are highly dependent on the purity of PEDF proteins prepared. However, it is also unlikely that the effects of PEDF on MOLT-3 T cells were non-specific because we have previously shown that [1]

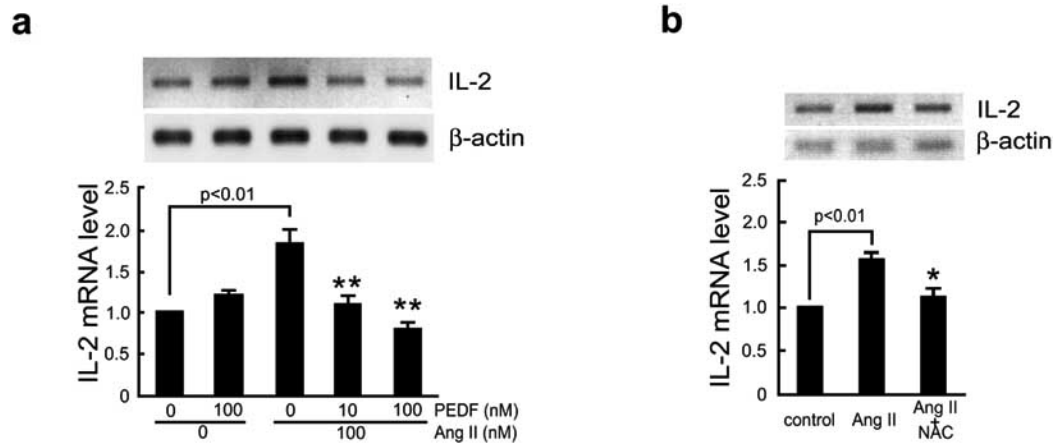

Fig. (2). Effects of PEDF or NAC on IL-2 mRNA levels in MOLT-3 T cells. Poly(A)$^+$RNAs were isolated from cells treated with or without 100 nM Ang II in the presence or absence of the indicated concentration of PEDF (a) or 10 mM NAC (b) for 6 h, and then analyzed by RT-PCR. Each lower panel shows the quantitative representation of IL-2 gene induction. Data were normalized by the intensity of β-actin mRNA-derived signals and related to the value of the control. *, P <0.05; **, P <0.01 compared to the value with Ang II alone. Similar results were obtained in three independent experiments.

purified PEDF proteins revealed a single band in a SDS-PAGE analysis [20] and [2] polyclonal Abs against PEDF neutralized the biological effects of PEDF on cultured ECs [6,7], and [3] we demonstrated here that same polyclonal Abs significantly reversed the effects of PEDF on IL-2 mRNA levels in MOLT-3 T cells.

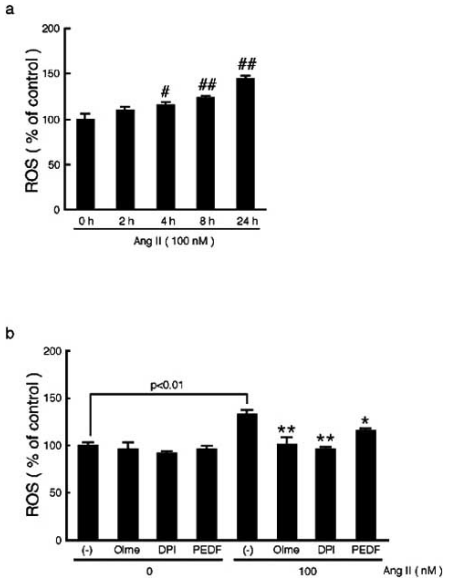

Fig. (3). Effects of PEDF on intracellular ROS generation in MOLT-3 T cells. (a) MOLT-3 T cells were treated with 100 nM Ang II for the indicated time periods. (b) MOLT-3 T cells were treated with or without 100 nM Ang II in the presence or absence of 100 nM PEDF, 100 nM olmesartan (Olme), or 50 nM DPI for 24 h, and then ROS were quantitatively analyzed. The percentage of ROS generation is indicated on the ordinate and related to the value for the control with no additives. #, P <0.05; ##, P <0.01 compared to the value without Ang II. *, P <0.05; **, P <0.01 compared to the value with Ang II alone.

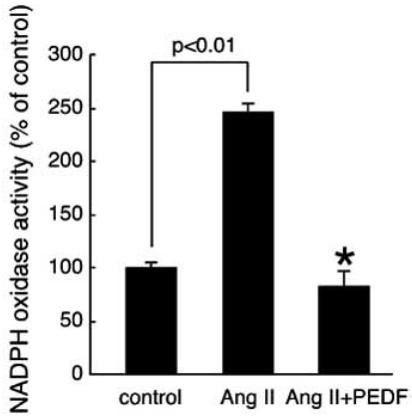

Fig. (4). Effects of PEDF on NADPH oxidase activity in MOLT-3 T cells. MOLT-3 T cells were treated with or without 100 nM Ang II in the presence or absence of 100 nM PEDF for 24 h, and then NADPH oxidase activity was measured by luminescence assay in 50 mM phosphate buffer containing 100 μM NADPH as the substrate. *, P <0.01, compared to the value with Ang II alone.

A chronic immune response involving proinflammatory T cell activation plays an important role in atherosclerosis [8,28]. In fact, T cells are present in atherosclerotic lesions at all stages of development [29]. They exhibit activation markers, and activated T cells are particularly prominent at the sites of unstable plaques [8,30]. Further, several lines of evidence suggest the involvement of Ang II in T cell activation in atherosclerosis; Ang II was not only prominently detected in T cell-rich regions of human atherosclerosis [31], but also elicited T cell activation and induced atherosclerotic plaque vulnerability in apolipoprotein E knockout mice [13]. In this study, we did experiments using MOLT-3 T cells, an immortalized T cell line, but not normal T cells from the circulation. Therefore, we do not know whether normal T cells within the circulation could respond in exactly same way as MOLT-3 T cells. However, these observations support the concept that suppression of T cell growth by PEDF could be clinically relevant; PEDF may slow the

development and progression of atherosclerosis by suppressing the Ang II-induced T cell activation *in vivo*.

Several reports showed that proliferation of T cells in response to mitogens *in vitro* was largely dependent on the production of IL-2 [32,33]. In concordance with these previous observations, we found here that Ang II promoted MOLT-3 T cells proliferation *via* autocrine production of IL-2. Since an Ang II type 1 receptor blocker, olmesartan completely blocked the growth-promoting effects of Ang II (Fig. **1b**), Ang II-type 1 receptor interaction-elicited ROS generation could be involved in IL-2 induction in MOLT-3 T cells, which was one of the molecular targets for PEDF. In support of this, ROS generation was reported to be required for T cell activation and subsequent IL-2 induction [34]. However, in the present study, 100 nM PEDF completely blocked the Ang II-induced up-regulation of IL-2 mRNA levels and subsequent increase in DNA synthesis (Figs. **1a** and **2a**), while it had a partial effect on ROS generation in Ang II-exposed MOLT-3 T cells (Fig. **3b**). These observations suggest that PEDF could block the mitogenic effects of Ang II on MOLT-3 T cells in a ROS-independent manner as well.

Petersen *et al*. recently reported that the estimated human blood concentration of PEDF was about 100 nM [35]. These observations suggest that the concentrations of PEDF used in these experiments were within a physiological range. Further, we have recently found that IL-2 expression was not an only target for PEDF in MOLT-3 T cells; PEDF at 100 nM blocked the Ang II-induced vascular endothelial growth factor (VEGF) expression in MOLT-3 T cells as well (unpublished data). Several reports have shown that VEGF-mediated angiogenesis plays an important role in plaque progression in atherosclerosis [36,37]. Therefore, taken together, our present study provides a novel beneficial aspect of PEDF on atherosclerosis. Blockade by PEDF of T cell growth and activation may become a novel therapeutic target for atherosclerosis.

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