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Laminin receptor mediates anti-inflammatory and anti-thrombogenic effects of pigment epithelium-derived factor in myeloma cells

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ABSTRACT

Pigment epithelium-derived factor (PEDF) has anti-inflammatory and anti-thrombogenic properties both in cell culture and animal models. Although adipose triglyceride lipase (ATGL) and laminin receptor (LR) are two putative receptors for PEDF, which receptor mainly mediates the beneficial effects of PEDF is largely unknown. In this study, we addressed the issue. siRNA raised against LR (siLR) and siATGL transfection dramatically decreased LR and ATGL levels in human cultured myeloma cells, respectively. Ten nM PEDF significantly reduced vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), intercellular cell adhesion molecule-1 (ICAM-1) and plasminogen activator inhibitor-1 (PAI-1) mRNA levels in siCon- or siATGL-transfected myeloma cells, whereas PEDF increased *rather than* decreased these gene expressions in siLR-transfected cells. Neutralizing antibody directed against LR (LR-Ab) or LR antagonist actually bound to LR and reduced mRNA levels of VEGF, MCP-1, ICAM-1 and PAI-1 in myeloma cells. Further, pre-treatment of LR-Ab or LR antagonist suppressed the binding of PEDF to LR and resultantly blocked the effects of PEDF in myeloma cells. In addition, high concentration of LR agonist mimicked the actions of PEDF on these gene expressions in myeloma cells. This study indicates that PEDF causes anti-angiogenic, anti-inflammatory and anti-thrombogenic reactions in myeloma cells through the interaction with LR. Target domain of LR agonist and antagonist might be involved in the PEDF-signaling to gene suppression in myeloma cells.

1. 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 [1]. PEDF is produced from a variety of tissues, including adipocytes, vascular and inflammatory cells [2], and has been shown to be an endogenous inhibitor of angiogenesis both in cell culture and animal models [3,4]. Moreover, we, along with others, have recently found that PEDF not only blocks advanced glycation end product– cytokine– or growth factor-induced endothelial cell damage, platelet aggregation, macrophage and T cell activation, but also inhibits hyperpermeability, inflammation, thrombus formation, and cardiovascular remodeling through its anti-oxidative and anti-inflammatory properties [5–15]. These observations suggest that PEDF could exert beneficial effects on vascular damage as well as tumor expansion and might be a novel therapeutic target for cardiovascular disease and cancers.

Adipose triglyceride lipase (ATGL) and laminin receptor (LR) are two putative receptors for PEDF [16,17]. The former receptor is required for the PEDF-induced lipolysis and triglycerides degradation in liver and adipocytes [18,19], whereas the latter mediates the anti-angiogenic activity of PEDF in endothelial cells [17]. However, which receptor is mainly involved in anti-inflammatory and anti-thrombogenic effects of PEDF is largely unknown. We have very recently found that PEDF could block vascular endothelial growth factor (VEGF)-induced proliferation and survival of human multiple myeloma cells through its anti-oxidative properties [20]. The findings suggest that cultured myeloma cells are one of the suitable cells for examining the PEDF actions *in vitro*. So, we examined here which putative PEDF receptors could mediate its anti-inflammatory and anti-thrombogenic properties in cultured myeloma cells.

2. Materials and methods

2.1. Materials

LR agonist, Lam.B1$_{925–933}$ and LR antagonist, murine epidermal growth factor fragment acetyl-(Cys(Acm)$_{33–42}$-amide (mEGF$_{33–42}$) were purchased from American Peptide Company, Sunnyvale, CA, USA and Bachem Americans, Inc. Torrance, CA, USA, respectively.

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Neutralizing antibodies (Abs) directed against LR (LR-Ab) from Abcam, Tokyo, Japan. Normal mouse IgG from Santa Cruz Biotechnology Inc. Dallas, TX, USA.

2.2. Preparation of PEDF proteins

PEDF proteins were purified as described previously [21]. 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).

2.3. Binding affinity of Lam.B1$_{925–933}$, mEGF$_{33–42}$, LR-Ab, and PEDF to LR

The binding affinity of Lam.B1$_{925–933}$, mEGF$_{33–42}$, LR-Ab, and PEDF to LR was measured using sensitive 27-MHz Quarts crystal microbalance (QCM) (Affinix Q; Initium, Tokyo, Japan) according to the method of Okahata et al. [22]. In brief, recombinant LR (Abnova, Taipei, Taiwan) was immobilized on a QCM surface through self-assembled monolayer of 16-mercaptohexadecanoic acid as described previously [23]. mEGF$_{33–42}$ (100 nM), or LR-Ab (13 nM) were added to the reaction vessel in the absence or presence of PEDF (100 nM), and the time course of the frequency decrease on the QCM was monitored. The binding amount of PEDF to LR was calculated from Sauebrey's equation [24]. According to this equation, 1 Hz frequency change corresponds to 0.62 ng/cm^2 of binding amount on the surface.

2.4. Cells

Human multiple myeloma cells, RPMI8226 (Human Science, Osaka, Japan) were cultured in RPMI1640 medium with 2 mM GultaMAX (Life Technologies Corporation, Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) containing 100 U/ml penicillin, and 100 g/ml streptomycin (Mediatech Inc., AK, USA). Experiments were carried out in a medium containing 1% FBS.

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

Sense and antisense human LR and ATGL siRNAs (siLR and siATGL) used in the experiments were chemically synthesized (Sigma Aldrich Japan K.K. Tokyo, Japan); sense and antisense of siLR were 5′-AACCUUCACUAACCAGAUCCAtt-3′ and 5′-UGGAUCUGGUUAGUGAAGGUUtt-3, respectively, and those of siATGL 5′-GAAUGUCAUUAUAUCCCACUUtt-3′ and 5′-AAGUGGGAUAUAAUGACAUUCtt-3′. Control non-silencing siRNAs (siCon) were obtained from Life Technologies Japan Ltd. (Silencer Negative Control #1 siRNA). Then the siRNA duplexes were transfected to myeloma cells using Lipofectamine RNAiMAX (Ilife Technologies Japan Ltd. Tokyo, Japan) as described previously [25]. After 4 days of transfection, LR, ATGL and α-tubulin protein levels were analyzed with Western blots.

2.6. Western blotting analysis

Proteins were extracted from siCon-, siLR and siATGL-transfected myeloma cells with lysis buffer as described previously [26]. Then the samples were separated by SDS–PAGE and transferred to nitrocellulose membranes. Membranes were probed with Abs against LR (Santa Cruz), ATGL (Abcam) or α-tubulin (Sigma, St. Louis, MO, USA), and then immune complexes were visualized with an enhanced chemiluminescence detection system (Amersham Bioscience, Buckinghamshire, UK).

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

siCon-, siLR, siATGL-transfected or non-transfected myeloma cells were treated with or without the indicated concentrations of PEDF in the presence or absence of 0–500 nM Lam.B1$_{925–933}$, 10 nM mEGF$_{33–42}$, or 10 mg/ml neutralizing LR-Ab 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 VEGF, monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), plasminogen activator inhibitor-1 (PAI-1), and 18S gene were Hs00900055_m1, Hs00234140_m1, Hs00164932_m1, Hs01126604_m1, and Hs03003631_g1, respectively.

2.8. Statistical analysis

All values were presented as mean ± standard error. Student's *t*-test or one-way analysis of variance followed Tukey's test was performed for statistical comparisons; $p < 0.05$ was considered significant.

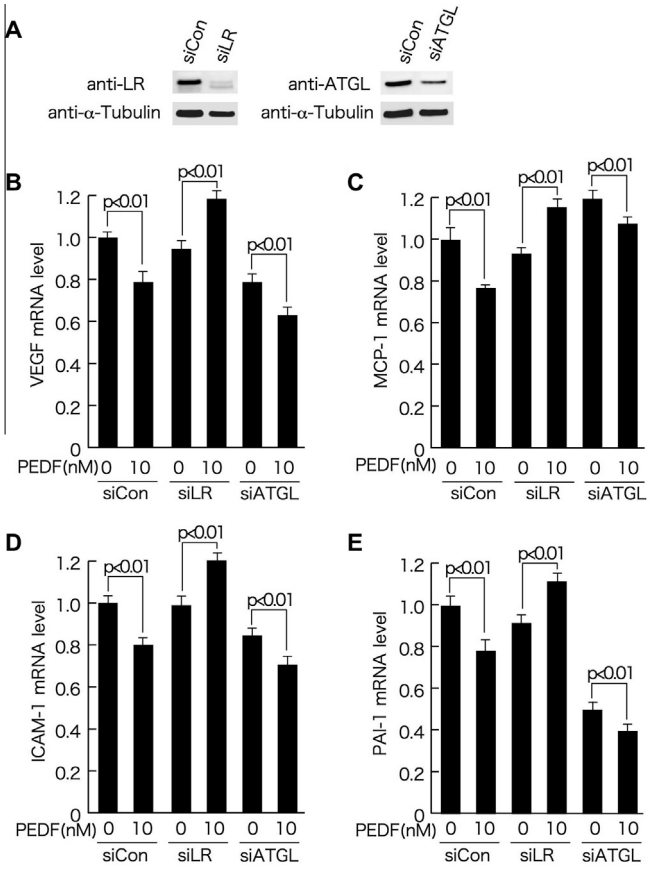

Fig. 1. Effects of siLR and siATGL on PEDF-exposed myeloma cells. siCon, siLR or siATGL were transfected to myeloma cells using Lipofectamine RNAiMAX. (A) After 4 days of transfection, LR, ATGL and α-tubulin protein levels were analyzed with Western blots. (B)–(E) siCon-, siLR, or siATGL-transfected myeloma cells were treated with or without 10 nM PEDF for 4 h. Then total RNAs were transcribed and amplified by real-time PCR. Data were normalized by the intensity of 18S mRNA-derived signals and then related to the value obtained with siCon treatment alone. $N = 3$ per group.