Journal of Dermatological Science 74 (2014) 150–158

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Cilostazol improves lymphatic function by inducing proliferation and stabilization of lymphatic endothelial cells

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ARTICLE INFO

Article history:
Received 11 September 2013
Received in revised form 25 December 2013
Accepted 6 January 2014

Keywords:
Cilostazol
Lymphedema
Lymphatic endothelial cells

ABSTRACT

Background: Cilostazol, an inhibitor of phosphodiesterase type III, is an antiplatelet agent and vasodilator. Some clinical reports have suggested that this drug can improve progressive and refractory lymphedema.
Objective: In this study, we investigated whether cilostazol has the potential to proliferate lymphatic vessels and to improve lymphatic function using human lymphatic endothelial cells (LECs) and mouse lymphedema models.
Methods: Human LECs were counted at several time points while they were cultured in the presence of cilostazol and/or protein kinase A inhibitor. After receiving a diet including 0.1% cilostazol or control diet, skin tissue and lymphatic function of *k-cyclin* transgenic (kCYC$^{+/-}$) mice, which have pernicious lymphatic dysfunction, was analyzed. A different lymphedema model was generated in wild type mice by excising circumferential tail skin to remove the superficial lymphatics. After oral administration of cilostazol, tail lymphedema was examined in this mouse model.
Results: Proliferation of LECs was promoted in a dose-dependent manner, which was partially inhibited by a protein kinase A inhibitor. Lymphatic vessel count increased in the cilostazol-treated kCYC$^{+/-}$ mice over that in the non-treated mice. Lymph flow improved in cilostazol-fed kCYC$^{+/-}$ mice as assessed by subcutaneous injection of Evans blue dye into the footpad. Oral administration of cilostazol also decreased lymphedema in a tail of wild type mice.
Conclusion: Cilostazol promoted growth of human LECs and improved lymph flow and lymphedema in two different mouse lymphedema models. These results suggest that cilostazol would be a promising agent for the treatment of lymphedema.

1. Introduction

Lymphedema is a progressive pathologic condition of the lymphatic system in which there is interstitial accumulation of protein-rich fluid, resulting in inflammation, adipose tissue hypertrophy, and fibrosis. Patients with lymphedema are prone to developing recurrent episodes of cellulitis due to increased microbial proliferation in the accumulated fluid. In some cases, chronic lymphedema causes rare cutaneous malignant tumors such as lymphangiosarcoma, which is known to be refractory to antineoplastic agents with poor prognosis [1,2]. Although lymphedema has been described for centuries, more attention has recently been paid to the disease because it is a relatively common complication in the treatment of malignancy [3]. No curative treatment for lymphedema is established. Acetylsalicylic acid (ASA), common anti-platelet medicine is empirically used to patients with lymphedema in some cases in Japan, although the clinical effect of ASA on lymphedema is to be examined [4]. It has been reported that transfer of the gene encoding vascular

Abbreviations: cAMP, cyclic AMP; PDE3, type III phosphodiesterase; eNOS, endothelial nitric oxide synthese; PKA, protein kinase A; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; LEC, lymphatic endothelial cells; kCYC$^{+/-}$, *k-cyclin* transgenic; WT, wild type; OD, optical density; LYVE-1, lymphatic vessel endothelial hyaluronic acid receptor 1; ASA, acetylsalicylic acid.

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0923-1811/$36.00
http://dx.doi.org/10.1016/j.jdermsci.2014.01.001

endothelial growth factor (VEGF)-C *via* viruses or plasmid achieved expectant results in animal model, but this approach is under investigation [5].

Cilostazol increases intracellular cyclic AMP (cAMP) levels by inhibiting type III phosphodiesterase (PDE3), resulting in anti-platelet aggregation and peripheral vasodilation [6]. This medicine is clinically used to improve intermittent claudication in patients with peripheral arterial disease and to prevent restenosis and target vessel revascularization in patients who undergo stenting of coronary or peripheral arteries [7]. Additionally, it has been reported that cilostazol induces nitric oxide production by activating endothelial nitric oxide synthese (eNOS) *via* a cAMP/protein kinase A (PKA)- and phosphatidylinositol 3-kinase/Akt-dependent mechanism and that this effect is involved in capillary-like tube formation in human aortic endothelial cells [8]. Shin et al. reported that cilostazol enhanced neovascularization in the hippocampus in a mouse ischemia model; the effect was induced by increased expression of eNOS and VEGF and recruitment of bone marrow-derived endothelial progenitor cells [9]. It has been reported that severe lymphorrhea and/or lymphedema of a lower extremity caused by chronic filariasis was improved after administrating cilostazol, suggesting that this drug can improve secondary lymphedema [4]. However, no study has been performed to determine what kind of effect cilostazol has on lymphatic endothelial cells (LECs) and whether cilostazol improves lymphedema.

We have recently generated transgenic mice expressing the Kaposi's sarcoma-associated herpesvirus latent-cycle gene, *k-cyclin* [10,11], under the control of the promoter of vascular endothelial growth factor receptor (VEGFR)-3, which is one of specific markers of lymphatic endothelium [12,13]. In Kaposi's sarcoma, *k-cyclin* is expressed by LECs and probably contributes to the edema within lesions [11,14]. Interestingly, the *k-cyclin* transgenic (kCYC$^{+/-}$) mice develop systemic lymphatic dysfunction such as progressive accumulation of chylous pleural fluid and dermal lymphedema. Immunohistochemistry has shown abnormal lymphatic architecture in these transgenic mice. Dermal edema in the skin and pleural effusion was detected by magnetic resonance imaging. In addition, the lymphatic drainage of injected contrast dyes was markedly impaired in these transgenic mice [12]. In the present study, we examined whether cilostazol could improve lymphatic dysfunction using kCYC$^{+/-}$ mice as lymphedema model. We also examined the effect of cilostazol on tail lymphedema of wild type mice *in vivo* and the effect of cilostazol on human LECs *in vitro*.

2. Materials and methods

2.1. Cell proliferation

Normal human dermal LECs were purchased from Lonza (Walkersville, MD). They were cultured with EBM-2 medium (Lonza) according to the manufacturer's instructions except for excluding VEGF or fibroblast growth factor (FGF). Cilostazol and ASA were purchased from Sigma–Aldrich (St. Louis, MO). Cilostazol was dissolved in 100% dimethyl sulfoxide (DMSO), whose solubility limit was 30 μM. LECs were cultured on 6 well plates in the presence of cilostazol (0, 1, 10, or 30 μM) or ASA (300 μM). Each well contained 0.1% DMSO as vehicle and 0.1% DMSO was used as control. Cultured LECs were treated with trypsin, and the number of cells was counted with a Coulter Counter (Beckman Coulter, Brea, CA). In addition, LECs were cultured on 96 well plates in the presence of cilostazol (0, 1 or 30 μM) and/or PKA inhibitor (14–22 Amide, Cell-Permeable, Myristoylated, Calbiochem, CA, 0, 1 μM). LEC proliferation was quantified with the colorimetric assay by measuring the conversion of a tetrazolium salt, WST-1 (Roche, Basel, Switzerland), into formazan by mitochondrial dehydrogeneases. The amount of formazan correlates to the number of metabolically active cells. Briefly, LECs were cultured for 48 h on 96 well plates. After adding the WST-1 solution, cells were incubated for 120 min. Optical density (OD) value was measured at 450 nm with a microplate reader (Bio-Rad).

2.2. Tubular formation and cell migration

To determine whether cilostazol would have any effects on tube-like structure of LECs, they were cultured on Matrigel (BD, Franklin Lakes, NJ) for 24 h after administration of cilostazol (0, 1 or 30 μM). Obtained images were analyzed using the tube formation analysis software Wimasis WimTube (Wimasis GmbH, Munich, Germany).

LECs were cultured on 6 well plates. When they were nearly confluent, grooves were made on the center of each well by scratching it with a sterile 9 mm wide cell scraper (Asahi Grass, Chiba, Japan). After the treatment, LECs were cultured in the presence of cilostazol (0, 1, 10, or 30 μM) and/or PKA inhibitor (0.2 or 1 μM). The cells were photographed 24 and 72 h after the treatment. From the photographs, the distance between migrating cells was measured.

2.3. Transgenic mice with lymphatic dysfunction

FVB/N wild type (WT) mice were purchased from Clea Japan, Inc. (Tokyo, Japan). kCYC$^{+/-}$ mice were generated as previously described [12] and used as a lymphedema model. Mouse chow containing 0.1% cilostazol was manufactured by Oriental Yeast (Tokyo, Japan). Mice were fed with or without 0.1% cilostazol after weaning, and their survival rate was evaluated for 24 weeks. All mice were free of pathogenic bacteria and viruses. Animal experiments were approved by the Animal Committee of National Center for Global Health and Medicine.

2.4. Examination of lymphatic transport

Dye containing 5 mg/ml Evans blue (Sigma–Aldrich) was injected subcutaneously into the hind limb footpads of kCYC$^{+/-}$ transgenic mice that had been fed with/without 0.1% cilostazol for 4 weeks. The bilateral popliteal lymph nodes were harvested 10 min after injection. The dye drained into the lymph nodes was eluted in formamide for 24 h at room temperature. The amount of Evans blue was quantified with a microplate reader (Bio-Rad, Hercules, CA) and calculated by subtracting the OD value of the contralateral lymph node from the OD value of the ipsilateral one. All experiments were performed using mice between 7 and 10 weeks of age.

2.5. Mouse tail model of lymphedema

To investigate the influence of cilostazol on lymphedema without *k-cyclin* gene expression, we utilized established mouse tail lymphedema model with minor modifications. A 2-mm wide circumferential full thickness skin excision was performed 10 mm from the base of mouse tail to remove the lymphatics, while carefully avoiding any disruption of the deep lymphatic system and the lateral tail veins [15]. The wounds were covered with occlusive dressing. Photographs of edematous tails were taken every day, and the diameter of each tail was subsequently measured from the pictures.

2.6. Immunohistochemistry

The ear skin was harvested from the mice after they were sacrificed. These samples were fixed in 3.5% paraformaldehyde and