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Pigment epithelium-derived factor reduces the PDGF-induced migration and proliferation of human aortic smooth muscle cells through PPARγ activation

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ABSTRACT

Our previous study demonstrated that pigment epithelium-derived factor (PEDF) plays an important role in the proliferation and migration of human aortic smooth muscle cells (HASMCs). In the present study, we examined whether PEDF inhibited platelet-derived growth factor (PDGF)-stimulated HASMC migration and proliferation. PEDF dose-dependently reduced PDGF-induced HASMC migration and proliferation *in vitro* and also arrested cell cycle progression in the G0/G1 phase, and this was associated with decreased expression of cyclin D1, cyclin E, CDK2, CDK4, and $p21^{Cip1}$ and increased expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$. The antiproliferative and antimigratory effects of PEDF were partially blocked by the PPARγ antagonist GW9662, but not by the PPARα antagonist MK886. In *in vivo* studies, the femoral artery of C57BL/6 mice was endothelial-denuded and the mice injected intravenously with PEDF or vehicle. After 2 weeks, both the neointima/media area ratio and cell proliferation (proliferating cell nuclear antigen-positive cells) in the neointima were significantly reduced and again these effects were partially reversed by GW9662 pretreatment. Our data show that PEDF increases PPARγ activation, preventing entry of HASMCs into the cell cycle *in vitro* and reducing the neointimal area and cell proliferation in the neointima *in vivo*. Thus, PEDF may represent a safe and effective novel target for the prevention and treatment of vascular proliferative diseases.

1. Introduction

Vascular smooth muscle cells (VSMCs) within the media of adult arteries are normally quiescent, proliferate at a low frequency, and are arrested in the G0 phase of the cell cycle. After vascular injury, growth factors and inflammatory cytokines released from endothelial cells, white blood cells, and platelets induce VSMCs to migrate into the intima, where they proliferate and subsequently form a thickened intima. Reentry of VSMCs into, and progression through, the cell cycle play a pivotal role in the pathogenesis of atherosclerosis, postangioplasty/in-stent restenosis, transplant vasculopathy, and vessel bypass graft failure (Ross, 1993). Although the migration and proliferation of VSMCs has been attributed to many factors, platelet-derived growth factor (PDGF) is an important mitogenic and chemotactic regulator, since the use of blocking anti-PDGF antibodies inhibits neointimal formation in rat models of arterial injury (Ferns et al., 1991). The ability to block PDGF-elicited SMC migration and proliferation may therefore improve existing therapeutic strategies by limiting late cardiovascular complications, such as in-stent restenosis or bypass graft failure (Andres and Castro, 2003).

Pigment epithelium-derived factor (PEDF) is a secreted glycoprotein first identified as a neurotrophic factor purified from the culture medium of human retinal pigment epithelial cells (Tombran-Tink et al., 1991). It has been shown to be a highly effective inhibitor of angiogenesis in cell culture and animal models, inhibiting retinal endothelial cell growth and migration *in vitro* and suppressing ischemia-induced retinal neovascularization *in vivo* (Dawson et al., 1999). In addition, expression of PEDF by endothelial cells can inhibit neointimal hyperplasia after vascular injury and prevent occlusive thrombus formation through its antioxidative properties (Nakamura et al., 2007). Our previous study

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demonstrated that PEDF, released by endothelial progenitor cells, inhibits the migration and proliferation of VSMCs (Wang et al., 2009). These observations suggest that PEDF might exert beneficial effects on highly proliferative vascular responses, such as postangioplasty restenosis. However, it is unclear whether it has a direct effect on the cell cycle progression and proliferation of human aortic SMCs (HASMCs) and thus might prevent the development of highly proliferative vascular diseases. Cell cycle progression is regulated by cell cycle regulatory proteins and these are, in turn, regulated by the peroxisome proliferator-activated receptor γ (PPARγ) signaling pathway (Wakino et al., 2000). PPARγ forms heterodimers with another nuclear receptor, retinoid X receptor, which then bind to peroxisome proliferator response elements (PPREs) located in the regulatory domains of genes (Hamblin et al., 2009). Several studies have provided evidence for the involvement of activated PPARγ in the prevention of SMC migration and proliferation that otherwise leads to subsequent neointimal formation (Wakino et al., 2000; Goetze et al., 1999). We were therefore interested in examining the effect of PEDF on HASMCs stimulated by PDGF and whether PEDF affected the expression of cell cycle-related proteins, an important event in cell proliferation, and the PPARγ pathway. In addition, we examined its effects on intimal thickening and cell proliferation in a mouse model of vascular injury. Our results showed that PEDF attenuated cell proliferation both *in vitro* and *in vivo* and that PPARγ activation is involved in the antiproliferative and antimigratory effects of PEDF.

2. Materials and methods

An expanded Materials and methods section is described in detail in supplemental materials.

2.1. HASMC cultures

HASMCs (Cascade Biologics Inc., OR, USA) were grown in smooth muscle cell growth medium (M231) supplemented with 5% growth supplement.

2.2. Effect of PEDF and PDGF on cell viability

To evaluate the optimal concentration and cytotoxicity of PDGF or PEDF, the cell viability of HASMCs was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

2.3. HASMC migration assay

To examine the effects of PEDF on the migration of PDGF-treated HASMCs, a wound healing assay was performed.

2.4. Smooth muscle cell proliferation assay

To monitor DNA synthesis, BrdU (5-bromo-2′-deoxy-uridine) labeling was performed using a kit from Sigma.

2.5. Cell cycle analysis

The DNA content and the movement of the cells through the mitotic cycle were analyzed by flow cytometry.

2.6. Western blot analysis

To prepare cell lysates, the cells were lysed for 1 h at 4 °C in 20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, pH 7.4. Western blot analyses were performed as described previously (Wang et al., 2009).

2.7. Immunocytochemistry

For immunocytochemistry, the cells were incubated with rabbit anti-human PPARγ antibodies. Bound antibody was detected by incubation with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibodies (Chemicon, CA, USA).

2.8. PPARγ luciferase activity assay

The PPAR-response element-containing luciferase reporter construct tk-PPREs-Luc was kindly provided by Ronald M. Evans (Salk Institute, San Diego, CA, USA).

2.9. Treatment with PPARγ small interfering RNA (PPARγ siRNA)

For transfection, 1 μg of PPARγ siRNA (Dharmacon, Chicago, IL, USA) was transfected into 10^6 HASMCs using 100 μL of Nucleofector Solution (Promega) and a Nucleofector II device (Promega).

2.10. Mouse femoral injury model and PEDF treatment

Male C57BL/6J mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan). All procedures were performed in accordance with the local institutional guidelines for animal care of the National Taiwan University and complied with the "Guide for the Care and Use of Laboratory Animals" NIH publication No. 86-23, revised 1985. For all surgical procedures, the mice were anesthetized by intraperitoneal injection of 50 mg/kg body weight of pentobarbital. Trans-luminal mechanical injury of the left femoral artery was induced basically according to the method of Sata et al. (2000). The mice were divided into 3 groups which were given: (1) 50 μL of PBS as a control, (2) 100 μg/kg body weight of mouse PEDF in 50 μL of PBS (Takenaka et al., 2008), and (3) 100 μg/kg of PEDF in 50 μL of PBS and 1 mg/kg of GW9662 (a PPARγ antagonist) (Cayman) (Collino et al., 2005); PEDF was given by intravenous injection into the femoral vein following the trans-luminal mechanical injury and GW 9662 was given by intraperitoneal injection 30 min before the injury.

2.11. Specimen collection, morphometric analysis, and immunohistochemistry

The mice were killed by intraperitoneal injection of an overdose of pentobarbital and the femoral artery was cut serial sections for morphometric analysis of neointima and the detection of cell proliferation.

2.12. Statistical analysis

All results are presented as the mean ± SEM. Differences in the data between groups were analyzed using the *t* test. A *P*-value < 0.05 was considered to indicate statistical significance.

3. Results

3.1. PEDF prevents the migration and proliferation of PDGF-treated HASMCs

Migration and proliferation of VSMCs in the intimal layer are pathological features of atherosclerosis and restenosis (Ross, 1993). We therefore tested the effect of PEDF on cell migration using a wound healing assay. At first, we tested the effects of the various