Effects of Osteopontin on Functional Activity of Late Endothelial Progenitor Cells

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ABSTRACT

The aim of this study is to investigate the effect of osteopontin (OPN) on functional activity of late endothelial progenitor cells (EPCs). Total mononuclear cells (MNCs) were isolated from human umbilical cord blood by Ficoll density gradient centrifugation, and then the cells were plated on fibronectin-coated culture plates. Late EPCs were positive for both 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (DiI-acLDL) and fluorescein-isothiocyanate-conjugated Ulex europaeus agglutinin lectin (UEA-1). Expression of von Willbrand factor (vWF) and kinase insert domain receptor (KDR) were detected by indirect immunofluorescence staining. Late EPCs of 3–5 passages were treated for 24 h with OPN (to make a series of final concentration: 0.005 µg/ml, 0.01 µg/ml, 0.05 µg/ml, 0.5 µg/ml, 2.5 µg/ml), or vehicle control. The proliferation, migration, and in vitro vasculogenesis activity of late EPCs were assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, modified Boyden chamber assay and an in vitro angiogenesis assay, respectively. Late EPCs adhesion assay was performed by replating cells on fibronectin-coated plates, and then adherent cells were counted. Incubation with OPN dose-dependently inhibited the proliferative, adhesive, and in vitro vasculogenesis capacity and increased migratory activity of late EPCs. J. Cell. Biochem. 112: 1730–1736, 2011.   © 2011 Wiley-Liss, Inc.

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Osteopontin (OPN) is a highly phosphorylated extracellular matrix glycoprotein that binds certain CD44 variants and integrin receptors [Katagiri et al., 1999]. Although OPN was originally identified as a matrix protein in bone, it has been found to be produced by many cell types including monocytes/macrophages, endothelial cells (ECs), and smooth muscle cells (SMCs) [O'Brien et al., 1994] and involved in many physiologic and pathologic processes, including cell adhesion [Reinholt et al., 1990], angiogenesis [Asou et al., 2001], apoptosis, inflammatory responses, and tumor metastasis [Denhardt et al., 2001].

Recently, OPN has emerged as a key factor in the development of atherosclerosis. Levels of OPN mRNA and proteins were elevated in atherosclerotic plaques [Ikeda et al., 1993; Giachelli et al., 1993]. In transgenic mice overexpressing the OPN gene, OPN overexpression was associated with a significant increase in medial thickening without injury and neointimal formation after arterial injury [Isoda

et al., 2002]. Moreover, OPN promoted early fatty-streak formation in OPN transgenic mice [Isoda et al., 2003]. Leali et al. [2007] reported that OPN up-regulation dramatically impaired re-endothelialization by inhibiting endothelial cell motility. Conversely, OPN blockade by neutralizing antibodies and OPN deficiency in OPN null mice prevented neointimal thickening and attenuated atherosclerosis [Liaw et al., 1997; Matsui et al., 2003]. Clinically, OPN plasma levels were significantly associated with the presence and the extent of cardiovascular disease independently of traditional risk factors [Ohmori et al., 2003]. These studies suggested that OPN plays a nonredundant role in atherosclerosis. However, the mechanism of how OPN induces atherosclerosis is ill understood.

Endothelial progenitor cells (EPCs), mainly derived from the bone marrow, can be mobilized to the peripheral circulation and have the capacity to circulate, proliferate, and differentiate into mature ECs. According to the time at which they appear in culture, there are two

different types of EPCs from circulating MNCs: early and late EPCs [Hur et al., 2004]. Although both EPCs are derived from MNCs, EPCs have different morphologies, growth patterns, and function in vitro [Hur et al., 2004; Yoon et al., 2005]. The early EPCs have low proliferative capacity and fail to form vessels while the late EPCs have a high proliferation rate and play a key role in neoangiogenesis in vivo.

Numerous studies have demonstrated that EPCs play important roles in endothelial repair, atherosclerosis, and angiogenesis. Almost all cardiovascular risk factors as well as various cardiovascular diseases are associated with EPCs impairment, both in number and function [Shantsila et al., 2007]. Moreover, reduced EPCs levels seem to be correlated with endothelial dysfunction [Heiss et al., 2005] and with an increased risk of cardiovascular events [Werner et al., 2005]. Reduced levels of circulating EPCs have been shown to be independent predictor of atherosclerotic disease progression [Schmidt-Lucke et al., 2005]. Estradiol (E2) has recently been recognized as an important regulator of EPCs number and function [Iwakura et al., 2003; Strehlow et al., 2003]. Moreover, Leen et al. [2008] demonstrated that E2 acceleration of the endothelial repair required OPN, both for bone marrow-derived cell recruitment and for endothelial cell migration and proliferation. It suggests that OPN has an affirmative action on EPCs. However, the effect of OPN on late EPCs has not been reported. Here we investigated the functional activity of late EPCs exposed to OPN in this study.

MATERIALS AND METHODS

ISOLATION AND CULTURE OF LATE EPCS

Human umbilical cord blood samples (20–70 ml) from healthy newborns were collected with informed consent. Late EPCs were cultured according to previously described techniques [Lin et al., 2000]. Briefly, total MNCs were isolated from umbilical cord blood by Ficoll density gradient centrifugation. Cells were plated on six-well plates coated with human fibronectin (Chemicon, Billerica, MA, USA) and maintained in endothelial cell basal medium-2 (EBM-2; Lonza, Walkersville, MD, USA) supplemented with EGM-2 MV single aliquots (Lonza) consisting of 5% fetal bovine serum, vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (b-FGF), epidermal growth factor, insulin-like growth factor-1, ascorbic acid, gentamicin sulfate, amphotericin-B, and hydrocortisone. After 24 h of culture, non-adherent cells and debris were removed by washing with EGM-2 medium, new medium was applied. Medium was changed daily for 7 days, and then every other day until the first passage. For all assays, late EPCs were used at passages 3–5.

CELLULAR STAINING

Fluorescent chemical detection of late EPCs was performed on attached cells. Direct fluorescent staining was used to detect dual binding of fluorescein-isothiocyanate (FITC)-conjugated Ulex europaeus agglutinin lectin (UEA-1; Sigma–Aldrich, St. Louis, MO, USA) and 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (DiI-acLDL; Invitrogen, Grand Island, NY, USA). Cells were first incubated with 10 μg/ml DiI-acLDL in EGM-2 medium for 3 h at 37°C and later

fixed with 2% paraformaldehyde for 10 min. After washing, the cells were incubated with UEA-1 (10 μg/ml) for 1 h. Cells were examined for uptake of DiI-acLDL and binding of UEA-1 using an inverted fluorescent microscope.

To detect cell-surface expression of von Willbrand factor (vWF) and KDR, cells were fixed in 2% paraformaldehyde for 10 min at room temperature, washed, and permeabilized for 10 min with 0.2% Triton X-100. After blocking with 10% goat serum for 30 min, cells were stained with polyclonal Rabbit anti-vWF antibody (sc-14014, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-KDR (sc-504, Santa Cruz) in a 1:100 dilution overnight followed by incubation in the dark with polyclonal anti-rabbit IgG FITC-conjugated secondary antibodies (goat; Santa Cruz) in a 1:100 dilution for 2 h. Images were obtained with an inverted fluorescent microscope.

EPCS PROLIFERATION ASSAY

Late EPCs proliferation was determined with an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Late EPCs of 3–5 passages were digested with 0.25% trypsin and then cultured in 96-well culture plate (200 μl/well). Late EPCs were treated with recombinant human OPN (R&D Systems Inc., Minneapolis, MN, USA; to make a series of final concentration: 0.005, 0.01, 0.05, 0.5, 2.5 μg/ml) or vehicle control. After being cultured for 24 h, late EPCs were supplemented with 10 μl MTT (5 g/l, Sigma–Aldrich) and incubated for another 4 h. Then the supernatant was discarded by aspiration and the late EPCs preparation was shaken in 150 μl dimethyl sulfoxide (DMSO) for 10 min, before the OD value was measured at 490 nm.

MIGRATION ASSAY

Late EPCs migration was evaluated by using a modified Boyden chamber assay. In brief, late EPCs were detached with 0.25% trypsin, and then 2×10^4 EPCs in 200 μl EBM-2 were seeded in the upper chamber of a transwell cell culture insert (8 μm pore size; BD Biosciences, Bedford, MA, USA). VEGF (50 ng/ml, Peprotech, Rocky Hill, NJ, USA) in EBM-2 was placed in the lower chamber. After incubation for 24 h at 37°C, the upper side of the membrane was wiped gently with a cotton ball. Then, the membranes were washed with phosphate buffer solution (PBS) and fixed with methanol. For quantification, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) solution. Cells migrating into the lower chamber were counted manually in five random high-power (×100) microscopic fields, and the average numbers of cells/100× field were determined.

CELL ADHESION ASSAY

Late EPCs were washed with PBS and gently detached with 0.25% trypsin. After centrifugation and resuspension in EGM-2, identical cell numbers were replated onto fibronectin-coated culture dishes and incubated for 30 min at 37°C. Adherent cells were counted by independent blinded investigators.

IN VITRO VASCULOGENESIS ASSAY

Analysis of capillary formation in matrigel (BD Biosciences) was performed according to the manufacturer's protocol. Matrigel (30 μl) was aliquoted into a 96-well plate and incubated at 37°C for

60 min. After trypsinization, 1×10^4 late EPCs were suspended with 100 μl culture medium and plated onto the pre-incubated matrigel. After incubation for 3 to 9 h, tube formation in the matrigel was observed under a microscope and the length of capillary tubes in a random field of each well was quantitated.

STATISTICAL ANALYSIS

All data are presented as mean ± SD from at least four independent experiments. Differences between group means were assessed by an unpaired Student's *t*-test for single comparisons or by ANOVA for multiple comparisons using SPSS 16.0. Values of $P < 0.05$ were considered significant.

RESULTS

CHARACTERIZATION OF LATE EPCS

Late EPCs appeared after 1–2 weeks as small colonies (Fig. 1A) in cultures of MNCs from the human umbilical cord blood and developed cobblestone-like cell morphology over time. During passaging the cells retained an endothelial-like morphology and formed cobblestone-like monolayers (Fig. 1B). Fluorescence microscopy showed that late EPCs were positive for both DiI-acLDL and UEA-1 (Fig. 1C–E). Immunophenotyping revealed that late EPCs expressed endothelial cell-surface antigens vWF (Fig. 1F) and KDR (Fig. 1G).

EFFECT OF OPN ON LATE EPCS PROLIFERATION

The effect of OPN on late EPCs proliferation was assayed using an MTT assay (Fig. 2). OPN dose dependently decreased late EPCs proliferative activity at OPN concentrations of 0.005 to 0.5 μg/ml, maximal at 0.5 μg/ml OPN (0.5 μg/ml OPN vs. control: 0.393 ± 0.008 vs. 0.453 ± 0.010, 490 nm light absorbance, $P < 0.01$). When the concentration of OPN was increased to 2.5 μg/ml, its inhibition on late EPCs proliferation decreased. Furthermore, late EPCs proliferative activity was significantly higher at OPN concentration of 2.5 μg/ml than 0.5 μg/ml (2.5 μg/ml OPN vs. 0.5 μg/ml OPN: 0.414 ± 0.004 vs. 0.393 ± 0.008, $P < 0.01$).
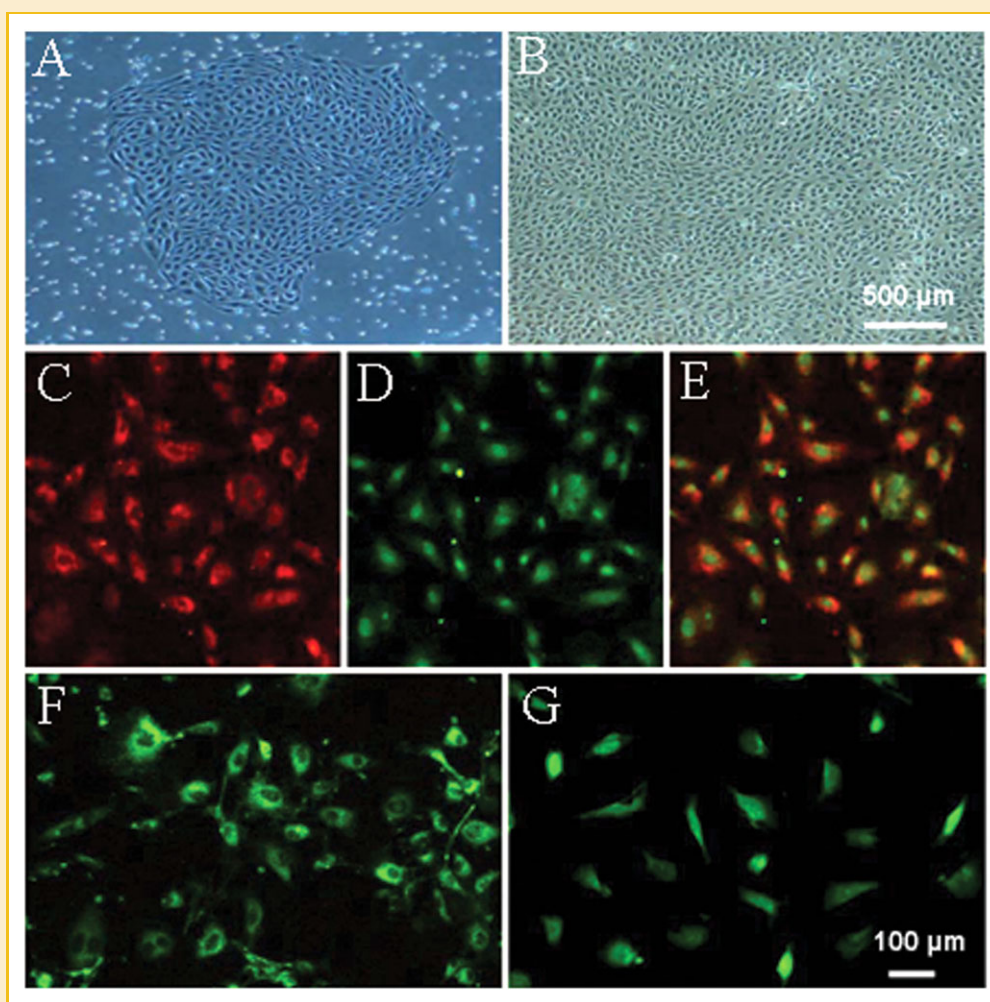

Fig. 1. Morphology and characterization of late EPCs from human umbilical cord blood. Small colonies appeared after 1–2 weeks in cultures of MNCs (A). Twenty-seven days after plating, late EPCs with cobblestone-like morphology were selected, passaged, and grown to confluence (B). Late EPCs were positive for both DiI-acLDL (C) and UEA-1 (D). Merged image of both stains (E). Immunofluorescence detection of vWF and KDR expression for late EPCs (F, G).
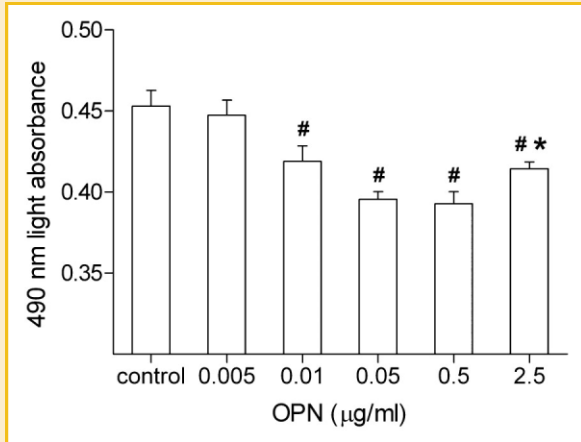
Fig. 2. Effect of OPN on late EPCs proliferation. OPN dose dependently decreased late EPCs proliferative activity with a peak at concentration of 0.5 μg/ml. Data are presented as mean ± SD. $n = 4$. # $P < 0.01$ vs. control. * $P < 0.01$ vs. OPN 0.5 μg/ml group.

EFFECT OF OPN ON LATE EPCS MIGRATION

The effect of OPN on late EPCs migration was analyzed in a modified Boyden chamber assay (Fig. 3). OPN profoundly improved cell migration at concentrations of 0.005 to 0.5 μg/ml, maximal at 0.5 μg/ml OPN (0.5 μg/ml OPN vs. control: 119.5 ± 6.1 vs. 69.5 ± 3.4, cells per 100× field, $P < 0.01$). When the concentration of OPN was increased to 2.5 μg/ml, its enhancement on late EPCs migration decreased.

EFFECT OF OPN ON LATE EPCS ADHESIVENESS

To study the possibility that OPN alters the adhesiveness of cultured human late EPCs, late EPCs were incubated with OPN for 24 h. After being replated on fibronectin-coated dishes, late EPCs pre-exposed to OPN exhibited a significant decrease in the number of adhesive cells at 30 min at OPN concentrations of 0.005 to 2.5 μg/ml (Fig. 4).
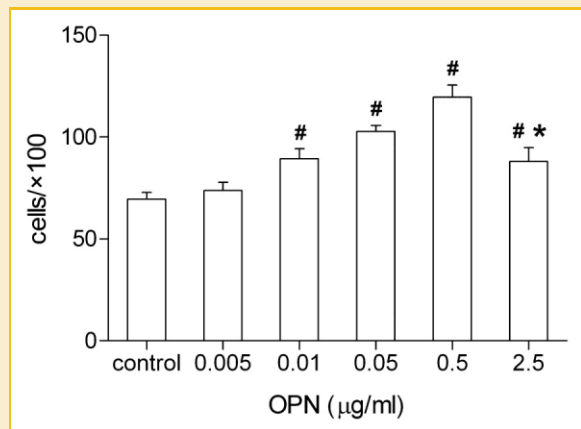

Fig. 3. Effect of OPN on late EPCs migration. OPN increased late EPCs migration activity in a concentration–dependent manner. Data are presented as mean ± SD. $n = 4$. # $P < 0.01$ vs. control. * $P < 0.01$ vs. OPN 0.5 μg/ml group.
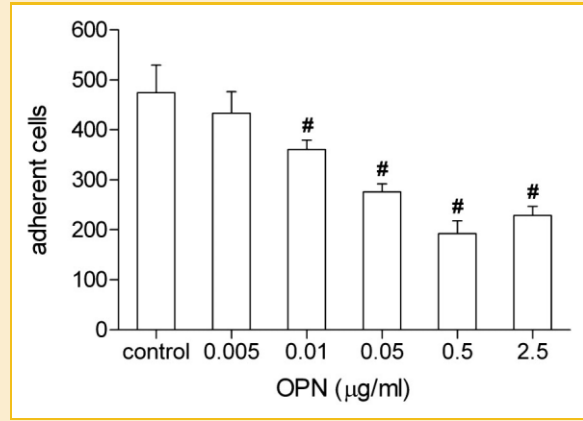

Fig. 4. Effect of OPN on late EPCs adhesive activity. OPN dose dependently decreased late EPCs adhesive activity with a peak at concentration of 0.5 μg/ml. Data are presented as mean ± SD. $n = 4$. # $P < 0.01$ vs. control.

The decrease in the number of adhesive cells occurred dose dependently, with a maximal effect achieved at 0.5 μg/ml (0.5 μg/ml OPN vs. control: 192.2 ± 25.7 vs. 474.0 ± 56.0 cells per 96 well, $P < 0.01$).

EFFECT OF OPN ON LATE EPCS VASCULOGENESIS

The effect of OPN on late EPCs vasculogenesis was evaluated by an in vitro vasculogenesis assay. After seeding the cells on a basement membrane matrix (Matrigel; BD Biosciences), late EPCs manifested tube formation (Fig. 5A). The response of late EPCs to OPN is depicted in Figure 5B–F. Tube length decreased in a dose-response manner to OPN concentrations (0.005 to 0.5 μg/ml) at 9 h of incubation (Fig. 5G), with peak production at 0.5 μg/ml OPN (0.5 μg/ml OPN vs. control: 18370.0 ± 1230.3 vs. 25879.9 ± 358.3, tube length (μm) per 96-well plates, 40× field, $P < 0.01$).

DISCUSSION

OPN, first identified in bone and involved in bone morphogenesis, is suggested to play a significant role in a variety of biological processes, including bone resorption, immune cell activation, inhibition of vascular calcification, and extracellular matrix remodeling. With respect to cardiovascular diseases, OPN is proposed as an important mediator of inflammation and implicated as a key factor in the development of atherosclerosis. It was reported that plasma OPN levels are higher in patients with coronary artery disease (CAD) than without CAD [Ohmori et al., 2003] and correlated with the severities of both coronary and aortic atherosclerosis [Momiyama et al., 2010]. In hypertensive patients plasma OPN levels are also elevated and can be decreased by angiotensin II receptor blocker and statins [Lorenzen et al., 2010].

Although the pathogenesis of atherosclerosis remains poorly understood, accumulating evidence suggested that endothelial damage and dysfunction played a major role. In recent years, it has become apparent that circulating EPCs are crucial in maintaining endothelial integrity and vascular homeostasis. It
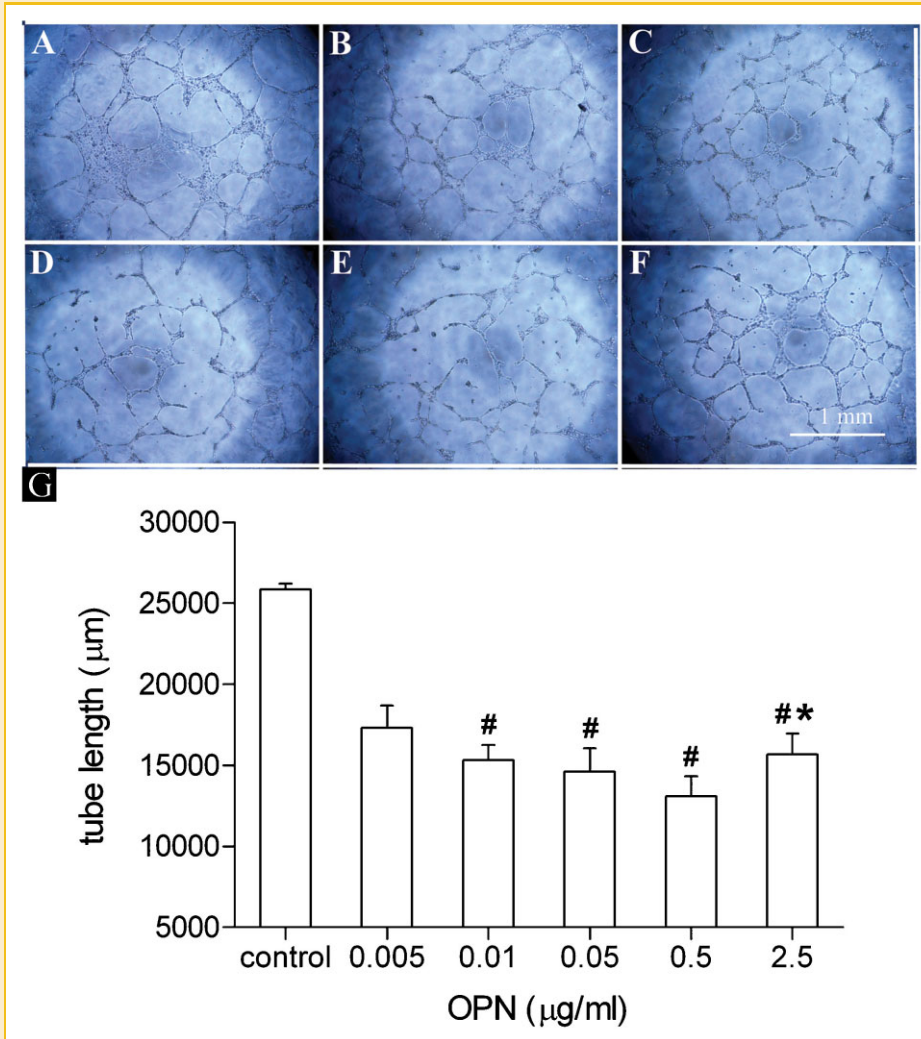
Fig. 5. Effect of OPN on late EPCs angiogenesis in vitro. Seeding of late EPCs on matrigel resulted in capillary network formation (A). OPN dose–dependently inhibited network formation: 0.005 μg/ml (B), 0.01 μg/ml (C), 0.05 μg/ml (D), 0.5 μg/ml (E), 2.5 μg/ml (F). Tube formation was determined by measuring the length of tubes (G). Data are presented as mean ± SD. $n = 4$. # $P < 0.01$ vs. control. * $P < 0.01$ vs. OPN 0.5 μg/ml group.

has been found that EPCs contributed up to 25% of ECs in newly formed vessels [Murayama et al., 2002; Suzuki et al., 2003]. Moreover, reduced EPCs levels seem to be correlated with endothelial dysfunction [Heiss et al., 2005] and with an increased risk of cardiovascular events [Werner et al., 2005]. EPCs can clearly be divided into two distinct populations: cytokine secreting early EPCs and vessel forming late EPCs [Hur et al., 2004], which have different roles in neovasculogenesis and vascular repair. Unlike early EPCs, late EPCs have been much less studied.

The result of the present study demonstrated that OPN could impair late EPCs proliferative, adhesive, and in vitro vasculogenesis capacity and promote migratory activity of late EPCs. Our result was not consistent with the data of Asou et al. [2001], which showed in vivo that the absence of OPN impaired angiogenesis. The result underlined the difference of OPN on the hemangiogenic endothelial cells between in vivo and in vitro studies. Given the well-established role of EPCs participation in neovascularization and re-endothe-

lialization, our results might suggest a novel mechanism of action of OPN in atherosclerosis: OPN decreases the proliferative, adhesive, and in vitro vasculogenesis activity of late EPCs, thus inhibits endothelial repair process, which contributes to endothelial dysfunction and subsequent to atherosclerosis.

It seems paradoxical that OPN enhanced late EPCs migratory activity but impaired the proliferative capacity of late EPCs, which had a positive and negative effect on endothelail repair, respectively. As a chemotactic factor, OPN is known to induce chemotactic movement of vascular SMCs, ECs, macrophages, and fibroblasts. On the other hand, the absence of OPN results in an increase in the number of hemopoietic stem and progenitor cell (HSC/HPC), both in bone marrow [Nilsson et al., 2005; Stier et al., 2005] and peripheral circulation [Grassinger et al., 2009]. Furthermore, exogenous OPN suppressed the proliferation and differentiation of primitive HSC/HPC in vitro [Nilsson et al., 2005]. Similar to these studies, our results suggest a potentially dual role of OPN in endothelial repair,

both protective through promoting migratory activity of late EPCs and detrimental through inhibiting proliferation of late EPCs.

In summary, we found that OPN impaired late EPCs proliferative, adhesive, and in vitro vasculogenesis capacity, which may be contributed to endothelial dysfunction and subsequent to arteriosclerosis. Our findings provide additional insight into the mechanism by which OPN promotes arteriosclerosis.

ACKNOWLEDGMENTS

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