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Role of α_v integrin in osteoprotegerin-induced endothelial cell migration and proliferation

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ABSTRACT

Osteoprotegerin (OPG) is a decoy receptor for the receptor activator of nuclear factor κB ligand (RANKL). However, the role of OPG in the endothelium remains unknown. In this study, we demonstrate that OPG stimulates the proliferation and migration of human microvascular endothelial cells (HMVECs). In addition, we show that treatment with integrin $\alpha_v\beta_3$ or integrin $\alpha_v\beta_5$ blocking antibody inhibits endothelial cell migration. In contrast, treatment with anti-$\alpha_v\beta_3$ antibody or anti-$\alpha_v\beta_5$ antibody alone did not inhibit OPG-induced proliferation. However, OPG-induced proliferation was inhibited when these antibodies were applied simultaneously. Furthermore, OPG evoked activation of extracellular signal-regulated kinase (ERK) 1/2, which has been linked to integrin α_v activity. Taken together, these results suggest that integrins $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ contribute to endothelial cell proliferation and migration induced by OPG.

Introduction

Osteoprotegerin (OPG), a member of the tumor necrosis factor (TNF) receptor superfamily, is involved in a variety of biological functions, including the regulation of bone turnover. OPG binds to the receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL), thereby neutralizing its function and negatively impacting osteoclast differentiation and survival (Simonet et al., 1997; Yasuda et al., 1998). Besides its role in bone metabolism, OPG has also recently been implicated in cardiovascular disease processes (Bennett et al., 2006). Knockout mice lacking OPG showed not only severe osteoporosis but also profound calcification of large arteries. OPG can also serve as a survival factor for endothelial cells (Malyankar et al., 2000), suggesting that it plays a role in vasculogenesis and the immune response, both of which are crucial for survival. However, the potential role of OPG in endothelial cell has not yet been fully investigated.

Recently, we reported that endothelial cells produce high levels of OPG in response to *Escherichia coli* lipopolysaccharide (Kobayashi-Sakamoto et al., 2004). Additionally, we showed that OPG treatment protects human microvascular endothelial cells (HMVECs) from detachment and apoptotic cell death induced by cysteine proteases produced by *Prophyromonas gingivalis*, an important pathogen of adult periodontitis (Kobayashi-Sakamoto et al., 2006). The processes governing detachment involve a complex interplay between various cell surface-associated components that regulate the proteolytic disruption of the extracellular matrix (ECM) and modify cell adhesion properties. Cell–ECM interactions are mediated by integrins, a family of adhesion receptors that attach the cell to both structural and matrix-immobilized proteins to promote cell survival, proliferation, and migration (Ruegg and Mariotti, 2003). In consideration of the above, we addressed in this report the possibility that integrins are involved in the OPG signaling pathway in the endothelium.

The purpose of the present study was (i) to further investigate the biological effect of OPG in endothelial cells, and (ii) to characterize the integrins and signaling pathways associated with OPG function.

Materials and methods

Reagents

Cell Counting Kit-8 and calcein-AM were purchased from Dojindo Laboratories (Kumamoto, Japan). Recombinant human osteoprotegerin (OPG) and recombinant human vascular endothelial growth factor (VEGF) were purchased from R&D Systems (Minneapolis, MN, USA). Antibodies to $\alpha_v\beta_3$ (L609) and $\alpha_v\beta_5$ (P1F6) integrins were purchased from Chemicon International (Temecula, CA, USA). A Cellular Activation of Signaling ELISA kit (CASE™) for ERK1/2 was obtained from SuperArray Bioscence (Frederick, MD, USA). The Falcon HTS FluoroBlock transwell system was obtained from Becton Dickinson (Franklin Lakes, NJ, USA). Human plasma fibronectin and vitronectin were purchased from Asahi Techno Glass Corporation (Tokyo, Japan).

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Biocoat Poly-D-Lysine Cellware 96-Well Plates, 96-well culture plates, and 25-cm^2 flasks were purchased from Becton Dickinson.

Endothelial cell culture

Human microvascular endothelial cells (HMVECs) were purchased from Clonetics (San Diego, CA, USA) and were cultured in endothelial cell growth medium-2MV (EGM-2M, Clonetics) according to the manufacturer's instructions. The cells were grown in 25-cm^2 flasks, passaged every 2 or 3 days, and used after 3 to 6 passages.

Assessment of cell proliferation

Vitronectin (10 μg/mL) or fibronectin (10 μg/mL) were used to pre-coat 96-well plates for 4 h at 37 °C. Nonspecific binding sites were blocked with 1% BSA. Subsequently, HMVECs were resuspended in human endothelial serum-free medium (H-SFM; Invitrogen, Carlsbad, CA) and seeded at a density of 1.5×10^4 cells/well onto non-coated or pre-coated plates with increasing doses of OPG (0, 20, 100 and 500 ng/mL). After 36 h incubation, relative cell proliferation was determined using Cell Counting Kit-8, in which 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) was used as a substrate. Briefly, after incubation for 36 h in the presence or absence of OPG, 10 μL of the kit reagent was added to each well, and the cells were incubated for an additional 3 h. Cell viability, determined by absorbance at 450 nm, was measured by scanning the plate with a microplate reader (Microplate Reader 680, Bio-Rad Laboratories, Hercules, CA). Each experimental condition was carried out in triplicate wells. For the inhibition experiment, cells were plated on poly-D-lysine-coated surfaces in H-SFM and allowed to adhere for 1 h. Subsequently, blocking antibodies targeting $\alpha_v\beta_3$ (L602) or $\alpha_v\beta_5$ (P1F61) were added. L602 and P1F6, and non-immune mouse IgG were used at 50 μg/mL. After a 1 h incubation period, OPG (500 ng/mL final concentration) was added to the medium. HMVECs were monitored by phase-contrast microscopy using an Olympus microscope. After an additional 22 h incubation, cell proliferation was assessed using a Cell Counting Kit-8.

Migration assay

Migration of HMVECs was assessed using a cell culture insert with a fluorescence blocking PET membrane (Falcon HTS FluoroBlock transwell system), according to the manufacturer's instructions. Briefly, HMVECs suspended in H-SFM were added to 3.0 μm FluoroBlock inserts, which were coated with vitronectin (10 μg/mL) or fibronectin (10 μg/mL), at a density 5×10^4 cells/insert. The lower chamber contained H-SFM and varying concentrations of OPG (20, 100, or 500 ng/mL). In certain experiments, the cells in the suspension were pretreated with blocking antibodies against $\alpha_v\beta_3$ or $\alpha_v\beta_5$ for 15 min; this was performed prior to the treatment with OPG. The cells were allowed to migrate across the inserts for 18 h at 37 °C. Cells that migrated to the lower side of the membrane were stained with calcein-AM (5 μg/mL) for 90 min at 37 °C. The fluorescence of cells that had migrated through the FluoroBlock inserts was read by a fluorescence plate reader (Infinite F200, Tecan Trading AG, Switzerland) equipped with bottom reading capabilities, at excitation/emission wavelengths of 485/530 nm. The extent of cell migration was judged from the amount of fluorescence detected in the lower chamber. Results were presented as relative fluorescence units (RFU).

ERK activation

The amount of activated (phosphorylated) ERK 1/2 protein relative to total ERK was quantified using a Cellular Activation of Signaling ELISA kit (CASE™; SuperArray Bioscence Frederick, MD, USA). The kit includes a complete antibody-based detection system for determining the ratio of the phosphorylated form of a specific protein and the total amount of the same protein. Briefly, HMVECs suspended in H-SFM (1.5×10^4 cells/well) were plated and allowed to adhere for 2 h. Subsequently, OPG (0, 100, or 500 ng/mL final concentration) or VEGF (20 ng/mL final concentration) was added to the medium. Cells were plated in 96-well clear flat bottom plates coated with poly-D-lysine, according to the manufacturer's instructions. After incubation for an additional 4 or 18 h, cells were fixed with 4% formaldehyde and probed with antibodies against phosphorylated ERK and total ERK, and then incubated with HRP-conjugated secondary antibodies. The

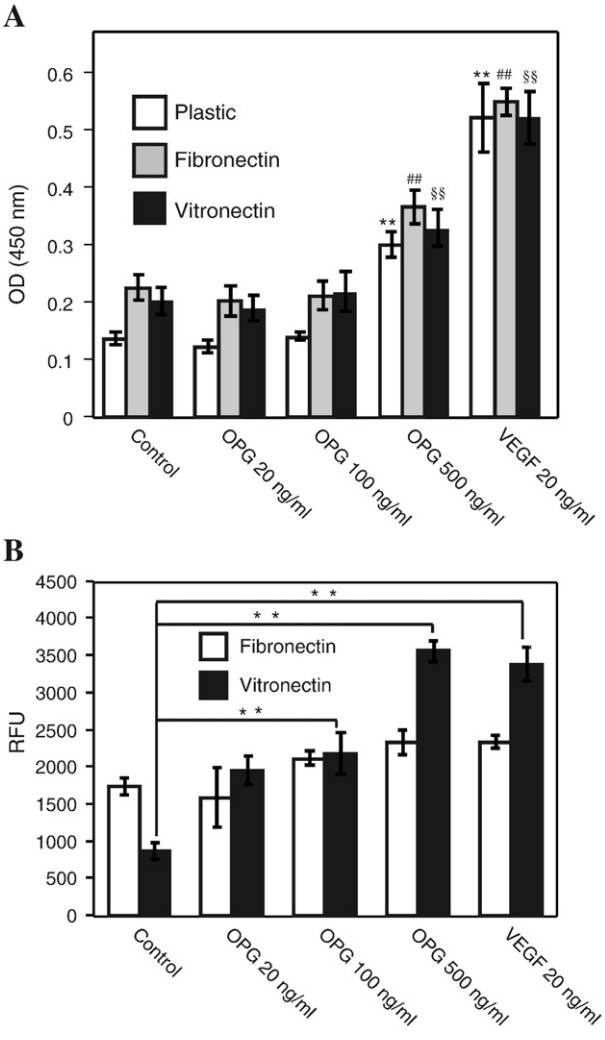

Fig. 1. OPG-induced proliferation and migration in HMVECs. (A) HMVECs were seeded into 96-well plates (1.5×10^4 cell/well) pre-coated with or without the indicated ECM proteins and incubated with medium alone (control) or with increasing doses of OPG (20, 100 and 500 ng/mL). After 36 h of incubation, relative cell proliferation was determined using a Cell Counting Kit-8. Treatment with VEGF (20 ng/mL) was used as a positive control. Data represent the means ± S.D. of triplicate wells from 1 of at least 3 replicated experiments. $^{**}P<0.01$ vs. control on plastic; $^{\#\#}P<0.01$ vs. control on plates pre-coated with fibronectin; $^{\S\S}P<0.01$ vs. control on plates pre-coated with vitronectin. (B) A chemotaxis assay using HMVECs (5×10^4 cell/insert) was performed on inserts pre-coated with fibronectin or vitronectin. The lower chamber contained H-SFM alone (control) or increasing doses of OPG (20, 100 and 500 ng/mL). Cells were allowed to migrate across the inserts for 18 h at 37 °C. Cells that migrated to the lower side of the membrane were stained with calcein-AM. The extent of cell migration was estimated from the fluorescence detected in the lower chamber. Results were presented as relative fluorescence units (RFU). Treatment with VEGF (20 ng/mL) was used as a positive control. Data represent the means ± S.D. of triplicate wells from 1 of at least 3 replicated experiments. $^{**}P<0.01$.