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Phloroglucinol Inhibits the *in vitro* Differentiation Potential of CD34 Positive Cells into Endothelial Progenitor Cells

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Abstract

Inhibiting the bioactivities of circulating endothelial progenitor cells (EPCs) results in significant inhibition of neovessel formation during tumor angiogenesis. To investigate the potential effect of phloroglucinol as an EPC inhibitor, we performed several *in vitro* functional assays using $CD34^+$ cells isolated from human umbilical cord blood (HUCB). Although a high treatment dose of phloroglucinol did not show any cell toxicity, it specifically induced the cell death of EPCs under serum free conditions through apoptosis. In the EPC colony-forming assay (EPC-CFA), we observed a significant decreased in the small EPC-CFUs for the phloroglucinol group, implying that phloroglucinol inhibited the early stage of EPC commitment. In addition, in the *in vitro* expansion assay using $CD34^+$ cells, treatment with phloroglucinol was shown to inhibit endothelial lineage commitment, as demonstrated by the decrease in endothelial surface markers of EPCs including $CD34^+$, $CD34^+/CD133^+$, $CD34^+/CD31^+$ and $CD34^+/CXCR4^+$. This is the first report to demonstrate that phloroglucinol can inhibit the functional bioactivities of EPCs, indicating that phloroglucinol may be used as an EPC inhibitor in the development of biosafe anti-tumor drugs that target tumor angiogenesis.

Key Words: Endothelial progenitor cell, Tumor angiogenesis, Phloroglucinol, Colony forming assay

INTRODUCTION

Tumor angiogenesis is a pathophysiological process that involves remodeling differentiated endothelial cells from pre-existing blood vessels during tumor growth (Folkman, 1976). Tumor angiogenesis allows nutritents and oxygen to be supplied to the cancer cells, which eventually promotes tumor cell proliferation and survival (Shweiki *et al.*, 1995; Toi *et al.*, 1995). Thus, this process is highly important to tumor development. Recently, several studies have reported that endothelial progenitor cells (EPCs) are closely related to tumor angiogenesis and can accelerate the growth of early tumors during tumor progression (Vajkoczy *et al.*, 2003; Li *et al.*, 2006). Therefore, screening of functional EPC inhibitors might be a potent and promising anticancer strategy, as well as identifying anti-angiogenesis agents that directly target cancer cells (Hicklin and Ellis, 2005; Fang *et al.*, 2007; Yi *et al.*, 2008).

Recently, plant-derived compounds have been screened for use as anti-cancer agents because these biomolecules cause few side-effects and have specific tumor cell targeting effects (Cragg and Newman, 2005). Phloroglucinol is a compound from *Ecklonia cava*, which is a species of brown alga found along the coast of Korea and Japan (Park *et al.*, 2011). Recently, it was show to display anti-inflammatory, antimicrobial, anti-allergic, antioxidant activity. In addition, this compound was reported to inhibit HIV-1 reverse transcriptase and protease activity (Kang *et al.*, 2006; Singh *et al.*, 2009; Gupta *et al.*, 2010). However, little is known about the effect of phloroglucinol on tumor angiogenesis via modulating EPC bioactivities.

Therefore, the aim of this study was to investigate whether phloroglucinol might be a potential EPC inhibitor that can prevent EPC commitment and/or differentiation.

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MATERIALS AND METHODS

Reagents

Phloroglucinol was purchased from Sigma-Aldrich (P1178). A solution of phloroglucinol was dissolved in dimethyl sulfoxide (DMSO, Amresco). The chemical structure of phloroglucinol is shown in Fig. 1A.

Culture of EPCs

EPCs were cultured according to a previously described technique (Ingram *et al.*, 2004). In brief, mononuclear cells (MNCs) were isolated from human umbilical cord blood (HUCB). HUCB samples (approximately 60 ml each) were collected from fresh placentas with attached umbilical cords, using density gradient centrifugation with a Ficol separating solution (Amersham). Freshly isolated MNCs were cultivated in 100 mm dishes coated with 1% gelatin (Sigma). The cultivation medium consisted of endothelial basal medium (EBM)-2 (Lonza, Walkersville, MD) supplemented with 5% fetal bovine serum (FBS), human basic fibroblast growth factor (bFGF), human vascular endothelial growth factor (hVEGF), human insulin-like growth factor-1 (hIGF-1), human epidermal growth factor (hEGF), ascorbic acid, and GA-1000 (complete EGM-2 medium). After 4 days, non-adherent cells were removed, and the tightly attached fraction of cells were re-plated and cultured for another 3 days. Cultivation was continued by replacing the EGM-2 medium until spindle-shaped colonies were formed after 14-21 days of culture. Medium was changed daily for 7 days and then every other day until the first passage.

Characterization of EPCs

Endothelial characteristics of the attached spindle-shaped cells were examined after uptake of Dil-conjugated Ac-LDL (Dil-Ac-LDL) (Biomedical Technologies Inc., MA, USA) and chemical binding with FITC-conjugated isolectin B4 (Sigma Chemical Co., WI, USA), a standard marker of endothelial lineage cells. EPCs were identified as the double-positive cells, using fluorescence microscopy. Immunophenotyping revealed that *ex vivo* cultured outgrowth ECs express several endothelial lineage markers, including CD31 and KDR/Flk-1; as well as several progenitor surface markers; CD34; and a pivotal functional marker, CXCR4, a receptor of SDF-1, which is involved in the homing of outgrowths of EPCs in ischemic sites.

Isolation of $CD34^+$ cells

HUCB was supplied by the Pusan National University Hospital. $CD34^+$ cells were isolated from human cord blood as reported previously (Suuronen *et al.*, 2006). Total MNCs were isolated by Ficol-gradient density centrifugation from cord blood. $CD34^+$ cells were separated from MNCs using CD34-bound microbeads and a magnetically activated cell sorter (Miltenyi Biotec, Bergisch-Gladbach, Germany) following the manufacturer's protocol.

Expansion of $CD34^+$ cells

$CD34^+$ cells (2×10^4) were expanded using the EPC expansion culture methods recently established in our laboratory. The harvested enriched $CD34^+$ cells were cultured in serum-free medium (StemSpan™; StemCell Technologies, Vancouver, BC, Canada) that was supplemented with 100 ng/ml stem cell factor (SCF), 50 ng/ml vascular endothelial growth factor (VEGF), 20 ng/ml interleukin-6 (IL-6), 20 ng/ml thrombopoietin (TPO), and 100 ng/ml fms-like tyrosine kinase-3 ligand (Flt-3L) for 7 days. After 7 days, floating cells were collected. In order to address the phenotype of surface markers expressed in CD34 cells expanded in the presence of phloroglucinol, fluorescence-activated cell sorting (FACS) analysis was carried out using a FACS Calibur analyzer (Becton Dickinson, San Jose, CA) and CellQuest™ Pro software (Becton Dickinson Immunocytometry Systems, Mountain View, CA). These cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD34 (BD Pharmingen, San Diego, CA), allophycocyanin (APC)-conjugated anti-c-kit antibodies (BD Pharmingen, San Diego, CA), phycoerythrin (PE)-conjugated anti-CD133 antibodies (Miltenyi Biotec, Auburn, CA, unless otherwise indicated), PE conjugated anti-KDR antibodies, PE conjugated anti-CD31 antibodies, PE conjugated anti-CXCR4 antibodies.

MTT assay for cytotoxic and apoptosis

Cell cytotoxic activity was determined using an MTT assay. In brief, *ex vivo* expanded EPCs (1×10^4 cells/well) were plated onto gelatin-coated, 96-well plates containing complete EGM-2 medium. After 24 h, the cells were serum starved in EBM-2

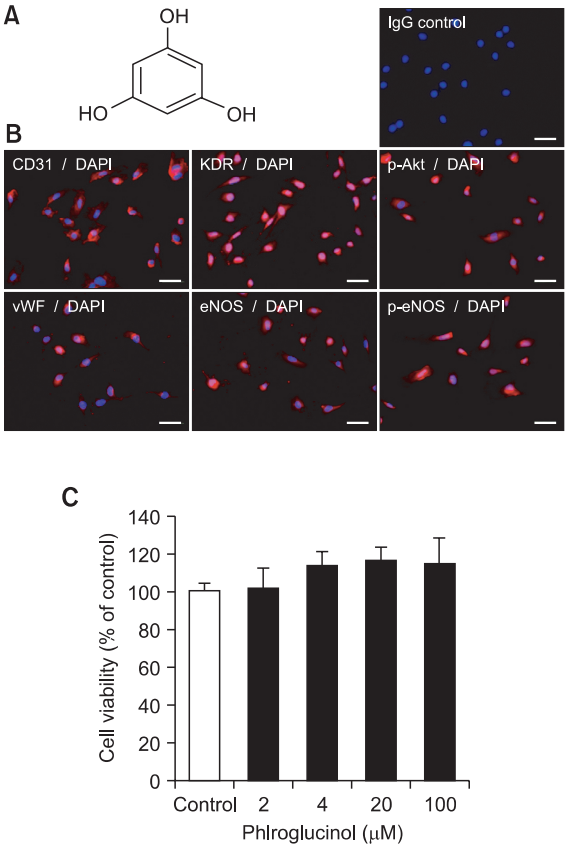

Fig. 1. Effect of phloroglucinol derivatives isolated from *Ecklonia cava* on cell toxicity. (A) Chemical structure of phloroglucinol. (B) Representative photomicrograps of *ex vivo* expanded EPCs, and immunostaining for endothelial cell (EC) markers, CD31, VEGFR2 (KDR) and von Willebrand factor (vWF), as well as pivotal markers of functional EPCs including eNOS, p-eNOS and p-Akt. Scale bar is 500 μm. (C) EPCs were treated with various concentrations of phloroglucinol and their cell viability was examined via the MTT assay after 24 h.

medium supplemented with 0.5% FBS for 12 h. The cells were then incubated with various concentrations of phloroglucinol in complete EGM-2 medium for 24 h and cell cytotoxicity was measured using the MTT assay.

To examine the effect of phloroglucinol on EPC apoptosis, *ex vivo* expanded EPCs (1×10^4 cells/well) were plated onto gelatinized 96-well culture plates in complete EGM-2 medium. After 24 h, the cells were cultured in serum-free EBM-2 medium for 12 h to induce apoptosis of EPCs. The cells were washed with EBM-2 medium containing 0.1% FBS and treated with different concentrations of phloroglucinol. The 0.1% FBS medium served as the vehicle control. After incubation for 24 h, the cells rewashed and subjected to the MTT assay.

Western blot analysis

Ex vivo expanded EPCs (1×10^6 cells/ml) were placed in a plate, and 24 h after plating, the cells were treated with various concentrations of phloroglucinol. The cells were harvested at the indicated times. Treated cells were then lysed with RIPA buffer (25 mM Tris · HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) containing a protease inhibitor cocktail (Thermo). Equal amounts of cell lysates were separated using 15% SDS-PAGE, which was then electrophoretically transferred to a polyvinylidene fluoride membrane (MILLIPORE), blocked with 5% nonfat milk, incubated with rabbit polyclonal antibody against cleaved caspase-3 (Cell signaling), and visualized with ECL reagents (Amersham).

EPC colony forming assay (CFA)

Human $CD34^+$ cells were cultured using methylcellulose-containing medium MethoCult (R) SF H4236 (Stemcell Technologies) containing 20 ng/ml stem cell derived factor (SCF), 50 ng/ml vascular endothelial growth factor (VEGF), 20 ng/ml interlukin-3 (IL-3), 50 ng/ml basic fibroblast growth factor (bFGF), 50 ng/ml epidermal growth factor (EGF), 50 ng/ml insulin-like growth factor-1 (IGF-1), 2 U/ml heparin, and 10% FBS on a 35 mm dish for 21 days. The cell density was 1.5×10^3 cells/dish. The EPC-CFUs were identified as large-EPC-CFUs and small-EPC-CFUs by microscope.

EPC-CFUs staining

After 21 days in culture, the EPC-CFU cells were washed with methylcellulose-containing medium and PBS and then treated with 2 μl/ml dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (Dil)-labeled acetylated low density lipoprotein (acLDL-Dil; Biomedical Technologies Inc. Stoughton, MA, USA) for 4 h. The cells were then fixed in 4% paraformaldehyde (PFA) for 30 minutes at room temperature. After washing with PBS, the cultures were reacted with fluorescein isothiocyanate (FITC)-labeled UEA-1 lectin (Sigma, St. Louis, MO) overnight at 4°C. After washing with PBS, the cultures were stained with DAPI for 30 minutes at room temperature. After washing with PBS, the EPC-CFUs were observed by fluorescence microscopy.

Statistical analysis

Statistical comparison of 2 groups was performed using the Student's t-test. The results were analyzed using the Statview 5.0 software package (Abacus Concepts, Inc., CA). The Scheffé's test was performed for multiple comparisons between each group after ANOVA. All data, which were obtained from at least 3 independent experiments, were expressed as means ± standard deviations.

RESULTS

Characterization of *ex vivo* expanded EPCs

EPCs were isolated and expanded *ex vivo* from HUCB mononuclear cells (MNCs). After obtaining informed consent, human umbilical cord blood was collected from healthy volunteers according to a protocol approved by the Ethics Review Board of the Hospital of the Pusan National University of Yangsan, Korea. To examine the characteristics of EPCs, immunophenotyping analysis was performed. As shown in Fig. 1B, *ex vivo* expanded EPCs expressed endothelial cell lineage antigens including CD31, VEGFR-2 (KDR), von Willebrand factor (vWF), as well as pivotal molecules of functional

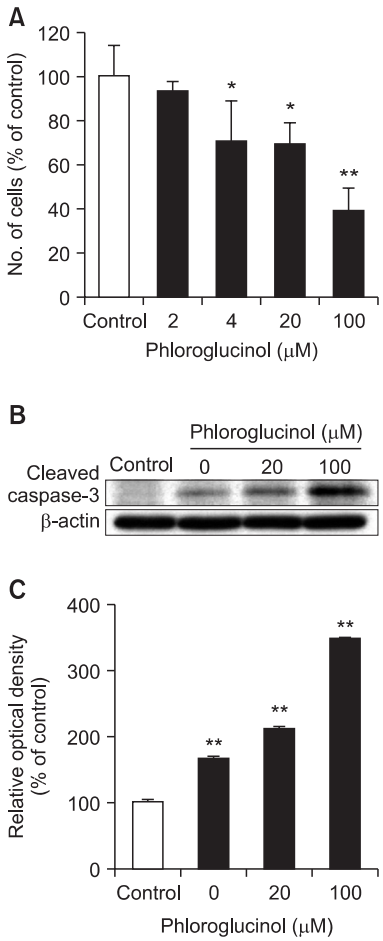

Fig. 2. Effect of phloroglucinol on the apoptosis of EPCs. (A) *Ex vivo* expanded EPCs were treated with different concentrations of phloroglucinol under apoptotic culture conditions in serum-free media for 24 h. The apoptosis index was measured in terms of cell numbers in the MTT assay. Phloroglucinol-induced apoptosis occurred in a dose-dependent manner (*p<0.05, **p<0.01). (B, C) Cleaved caspase-3, which is a typical indicator of apoptosis, was increased 3 h after treatment with phloroglucinol in a dose dependent manner, as measured using western blot analysis. The complete EGM-2 medium served as the normal control and 0.1% FBS medium served as the vehicle control (*p<0.05, **p<0.01).

EPCs, including eNOS, p-eNOS and p-Akt.

Effect of phloroglucinol on cell toxicity of EPCs

To evaluate the cytotoxicity of phloroglucinol against EPCs, the MTT assay was performed. As shown in Fig. 1C, phloroglucinol did not reduce cell viability in EPCs at doses below 100 μM of phloroglucinol. Therefore, phloroglucinol concentrations were varied from 2 to 100 μM to investigate the *in vitro* inhibitory effect of phloroglucinol against EPCs.

Effect of phloroglucinol on apoptosis of EPCs

To evaluate the effect of phloroglucinol on cell survival of EPCs, the MTT assay was performed using serum starved EPCs. In these experiments, the *ex vivo* expanded EPCs were cultured under serum-free culture conditions at different phloroglucinol concentrations and the cells were analyzed using the MTT assay. In these experiments, phloroglucinol was shown to promote cell death in a dose-dependent manner (Fig. 2A). To further evaluate the apoptotic effect of phloroglu-

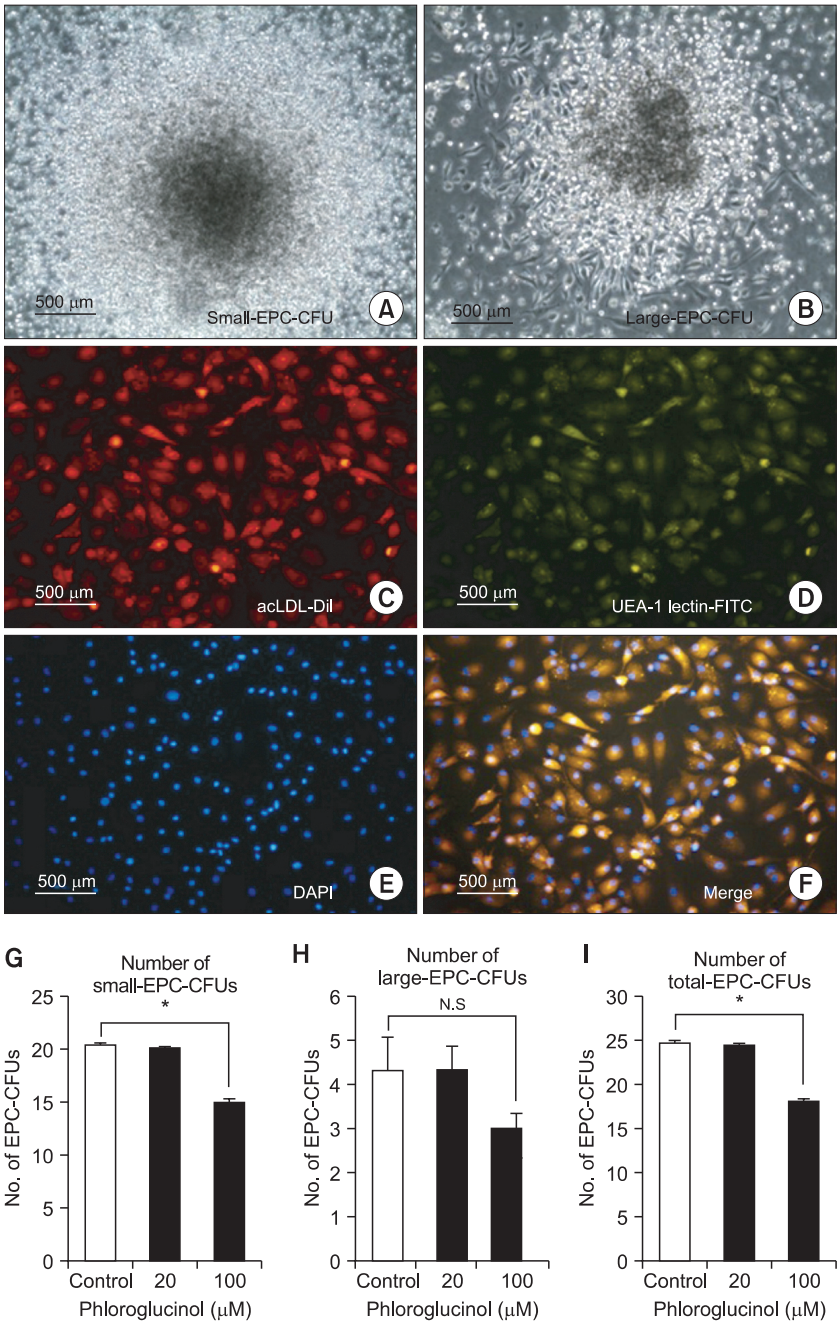

Fig. 3. Effect of phloroglucinol on production of EPC lineage cells of $CD34^+$ cells in EPC-CFA. (A, B) Representative image of small-EPC-CFUs and large-EPC-CFUs derived from HUCB-MNCs after 21 days. (C) Binding of acetyl LDL (acLDL)-DiI uptake (red), (D) UEA-1 lectin (green), (E) cell nuclei were stained with 4',6-diamidine-2-phenylidole dihydrochloride (DAPI) and (F) merge of C,D and E. Scale bar is 500 μm. Statistical bar graphs for Small-EPC-CFUs (G), large-EPC-CFUs (H) and total-EPC-CFUs (I) (*p<0.05).

cinol on EPCs, cleaved caspase-3 expression was measured by western blotting analysis. As shown in Fig. 2B, C, treatment with phloroglucinol induced apoptosis, which was demonstrated by the higher concentrations of cleaved caspase-3.

Effect of phloroglucinol on the EPCs colony forming ability

To determine whether phoroglucinol might inhibit the process of endothelial lineage differentiation of HUCB derived $CD34^+$ cells, EPC-CFA was utilized. As shown in Fig. 3A, B, EPC cells were capable of forming both small-EPC-CFUs and large-EPC-CFUs. Morphologically, small-EPC-CFUs were round-shaped, whereas large-EPC-CFUs were mainly spindle-shaped. Fluorescence immunocytochemial analysis was used to examine the EPC phenotype in the CFUs by measuring uptake of Dil-labelled AcLDL and FITC-conjugated UEA-1 lectin binding. EPCs were identified as double positive cells by fluorescence microscopy (Fig. 3C-F). In addition, the number of small-EPC-CFUs was significantly decreased in the phloroglucinol treated group (15 ± 4) relative to the control group (20.333 ± 2.309) (Fig. 3G); however, treatment did not affect the number of large-EPC-CFUs (Fig. 3H). Total-EPC-CFUs were significantly lower in the phlorogluciol treated group (18 ± 3.605) than the control group (24.666 ± 3.214) (Fig. 3I). These results indicated that phloroglucinol might affect EPC commitment, especially during the early stage of EPC lineage cells.

Phloroglucinol affects *ex vivo* expansion of $CD34^+$ cells

To examine the effect of phloroglucinol on specific serum free expansion of stem cells, we isolated cord blood-derived $CD34^+$ cells and expanded them as described in Materials and Methods (Fig. 4A). FACS analysis revealed that the freshly isolated $CD34^+$ cell population contained 95-98% $CD34^+$ cells and the expanded cells were 46.7% $CD34^+$ cells after 7 days. The cell number expanded by $7.35\times10^5 \pm 0.835\times10^5$ and the phloroglucinol treated $CD34^+$ cells expanded by $6.983\times10^5 \pm 1.079\times10^5$ and $7.7\times10^5 \pm 1.664\times10^5$. As shown in Fig. 4B, no significant difference in the number of expanded $CD34^+$ cells were observed, implying that phloroglucinol did not affect stem cell proliferation.

Effect of phloroglucinol on expression of surface markers on expanded cells

To evaluate the effect of phloroglucinol on stem cell expansion of $CD34^+$ cells, we further measured endothelial lineage markers including CD31 and KDR, as well as several progenitor surface markers, including CD34, CD133, CXCR4 and c-kit (Fig. 4C). As shown in Fig. 5, there was a drastic decrease

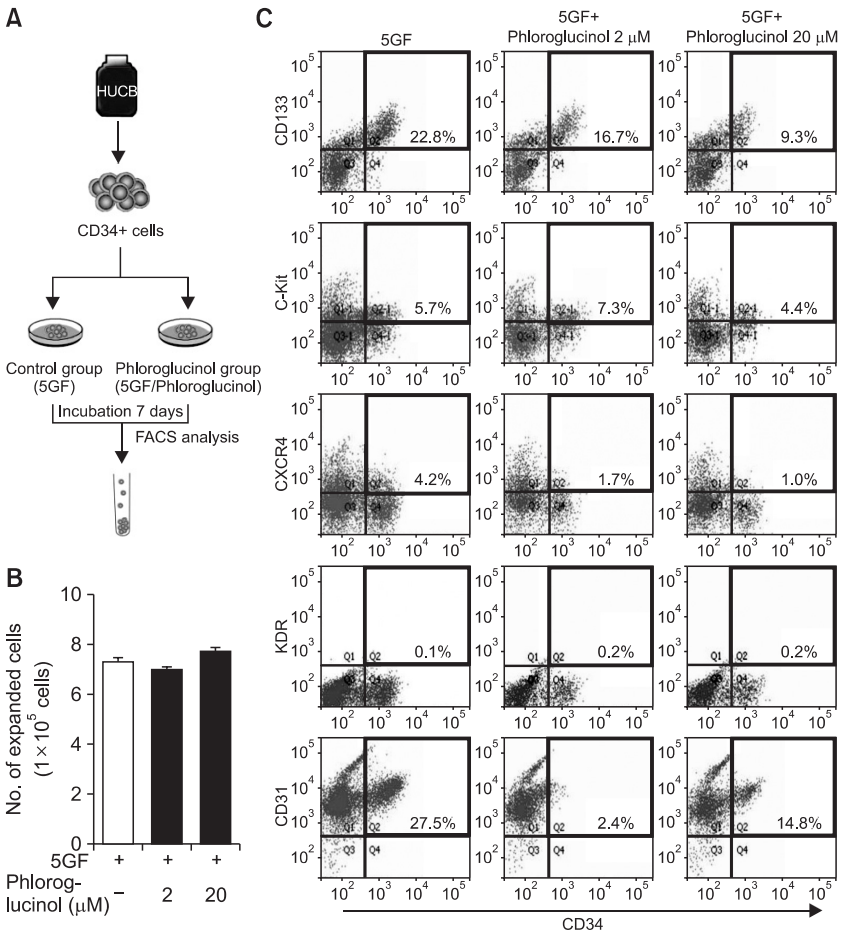

Fig. 4. Effect of phloroglucinol on stem cell expansion of $CD34^+$ cells. (A) Experimental scheme for the stem cell expansion of $CD34^+$ cells. (B) The expanded cell number of $CD34^+$ cells under *ex vivo* expansion culture conditions (SCF, TPO, VEGF, Flt3-L and IL6). (C) Expressional change in EPC surface markers for CD133, c-kit, CXCR4, KDR and CD31 after incubation with phloroglucinol.

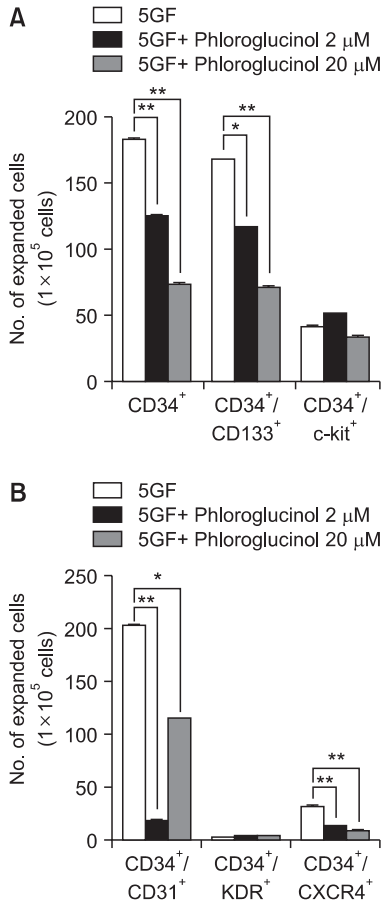

Fig. 5. Statistical analysis of effect of phloroglucinol on stem cell expansion of $CD34^+$ cells. (A) The number of $CD34^+$ and $CD34^+/CD133^+$ cells was significantly decreased in the phloroglucinol-treated group (*p<0.05, **p<0.01). (B) The number of $CD34^+/CD31^+$ and $CD34^+/CXCR4^+$ cells was decreased in the phloroglucinol-treat group (*p<0.05, **p<0.01).

in the number of CD34 positive cells. Similarly, there were significant decreases in the number of $CD34^+/CD133^+$ and $CD34^+/CD31^+$ cells after $CD34^+$ expansion when phloroglucinol (2 and 20 μM) was added to the culture media, although there was no significant difference in the number of $CD31^+/KDR^+$ and $CD34^+$/c-kit^+ cells. In addition, the number of $CD34^+/CXCR4^+$ expressing cells were significantly reduced, suggesting that phloroglucinol might affect EPC function during tumor angiogenesis, because the CXCR4/SDF-1 axis plays a pivotal role in homing to ischemic sites.

DISCUSSION

Screening plant-derived compounds for anti-cancer agents has recently received more attention, because treatment with these biomolecules results in few side-effects and these compounds may have strong cell targeting effects (Cragg and Newman, 2005). Therefore, in this study, we examined whether phloroglucinol can inhibit EPC bioactivities including EPCs lineage differentiation of $CD34^+$ cells and demonstrated, for the first time, that phloroglucinol can act as an EPC inhibitor using several *in vitro* EPC functional assays.

Recently, Masuda *et al.* developed an EPC-colony forming assay (CFA) to discriminate the differential potential of EPC commitment/differentiation (Masuda *et al.*, 2011). EPC-CFA can be clearly classified into two types of EPC colonies; small-EPC-CFUs, which are essentially clusters of immature and proliferative EPCs, and large-EPC-CFUs, which are clusters of further angiogenic potential EPCs (Kwon *et al.*, 2011; Masuda *et al.*, 2011). We first demonstrated that phloroglucinol significantly attenuated the onset of small-EPC-CFUs, suggesting that it might affect the early stage of EPC commitment.

We also examined the effect of phloroglucinol on stem cell expansion/proliferation of CB-derived $CD34^+$ cells. The addition of phloroglucinol to the stem cell culture media resulted in a significant reduction in the number of endothelial lineage cells, CD34/CD31, as well as stem/progenitor cell markers CD34, CD34/CD133, CD34/CXCR4. Thus, phloroglucinol was shown to decrease the expression of surface antigens on functional EPCs, and due to the decreased number of $CD34^+$ cells, $CD133^+$ cells and/or $CXCR4^+$ cells after treatment, phloroglucinol might affect neovessel formation of EPC during tumor angiogenesis (Prater *et al.*, 2007).

Based on the combined results presented in this study, phloroglucinol holds great promise for use as an anti-angiogenesis drug against tumor angiogenesis.

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