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Ginsenoside Rg3 Inhibits Endothelial Progenitor Cell Differentiation through Attenuation of VEGF-Dependent Akt/eNOS Signaling

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Endothelial progenitor cells (EPCs) play a critical role both in vascular repair after cell transplantation for ischemic diseases and in the growth of early tumors by intervening with the angiogenic switch during tumor progression. This paper reports on the effect of ginsenoside Rg3 in EPCs as a candidate angiogenesis inhibitor for *in vitro* functional assays. $CD34^+$ cells were isolated from human cord blood and the study investigated whether or not ginsenoside Rg3 regulated EPC bioactivities including cell proliferation, differentiation, migration and tube formation. Although ginsenoside Rg3 did not affect the *ex vivo* expansion of CD34 and/or KDR (VEGFR2) stem/progenitor cells, treatment with ginsenoside Rg3 led to a significant decrease in CD34-expressing cells, specifically the absolute number of expanded $CD34^+$ cells. Importantly, a significantly decreased number of EPC colony-forming units among human cord blood-derived $CD34^+$ cells was observed, implying that ginsenoside Rg3 inhibited EPC differentiation, in particular, the commitment to primitive EPC colonies (the early stage of EPC differentiation). Moreover, treatment of CD34-derived EPCs with ginsenoside Rg3 resulted in the attenuation of VEGF-dependent Akt/eNOS signaling as well as the inhibition of migration and tube formation. In conclusion, this study provides *in vitro* evidence for ginsenoside Rg3 as a potential therapeutic molecule, specifically as an angiogenesis inhibitor that functions by attenuating EPC bioactivities.

Keywords: adult stem cells; differentiation inhibitor; endothelial progenitor cells; angiogenesis inhibitor; ginsenoside.

Supporting information may be found in the online version of this article (Supplementary Material)

INTRODUCTION

Ginseng has been used in Asian countries for thousands of years as a folk medicine to treat various diseases owing to its wide range of pharmacological and therapeutic actions, such as biomodulation, antihypoxia stress and antiaging activities in the central nervous system, the immune system and the cardiovascular system. The effects of ginseng include general 'tonic', antifatigue, antistress, immunomodulatory and anticancer (Liu and Xiao, 1992; Surh *et al*., 2001). The current data indicate that the major biologically active component of ginseng, ginsenoside Rg3, inhibits CD34 stem cell differentiation and/or commitment to endothelial progenitor cells (EPCs).

Endothelial progenitor cells play an important role in early tumor growth by intervening with the angiogenic switch; EPCs promote tumor neovessel formation through the production of angiogenic cytokines during tumor progression. They also play an important role in the restoration of ischemic vascular disease (Asahara *et al*., 1999; Gao *et al*., 2008; Kopp *et al*., 2006; Rafii and Lyden, 2003). The newly forming vessels not only help to meet the growing metabolic demands of the tumor by supplying additional nutrients, but also provide potential routes for tumor dissemination and metastasis (Abe, 2008; Gupta and Qin, 2003). Angiogenesis continues as the tumor continues to develop. Tumor–stroma interaction enhances neovascularization through angiogenic factors, such as vascular epithelial growth factor (VEGF) (Ferrara, 2000; Nagy *et al*., 2009; Saharinen *et al*., 2011) and activated Akt/eNOS signaling. As a result of these signals, tumor angiogenesis occurs via proliferation, migration and differentiation of ECs and/or EPCs (Feliers *et al*., 2005; Somanath *et al*., 2006; Yi *et al*., 2008). Because angiogenesis is necessary for tumor growth and metastasis, inhibition of angiogenesis is an anticancer strategy (Gasparini *et al*., 2005; Kerr, 2004). However, despite these findings, effective therapeutic targeted biomolecules that inhibit tumor angiogenesis promoted by EPCs have not yet been elucidated.

Recently, Masuda *et al*. developed a CFU-EPC assay system to elucidate the EPC differentiation cascade. They reported two types of attaching cell colonies composed of small or large cells with endothelial lineage potential and properties, termed small EPC colony-forming units (with primitive EPC characteristics) and

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large EPC colony-forming units (with definitive EPC characteristics), respectively (Masuda *et al*., 2012). An EPC-CFU assay system will be very useful in order to search for functional inhibitors of EPCs, because it also can reveal changes in the differential cascade due to specific molecules, as well as changes in the number of progenitor cells, and it is possible to perform clonogenic assays using freshly isolated individual stem cells.

Accumulating evidence suggests that ginsenoside Rg3 inhibits tumor growth by inhibiting the invasion and metastasis of various tumors, including Lewis lung carcinoma (Yi *et al*., 2005; Zhang *et al*., 2006), intestinal adenocarcinomas and B16 melanoma (Iishi *et al*., 1997; Liu *et al*., 2004), as well as suppressing the proliferation of prostate cancer cells. However, little is known about the effect of ginsenoside Rg3 on tumor angiogenesis via EPC differentiation. Therefore, this study examined whether ginsenoside Rg3 is a targeted therapeutic biomolecule for the suppression of EPC commitment and/or differentiation. This study demonstrated that treatment of cord blood (CB)-derived CD34 stem/progenitor cells with ginsenoside Rg3 inhibits the onset of the early stages of EPC colonies and delays EPC differentiation by attenuating Akt/eNOS signaling *in vitro*.

MATERIALS AND METHODS

Drugs. Ginsenoside Rg3 was purchased from BTGin Co., Ltd (Okcheon, Chungbuk, South Korea). A solution of Rg3 was freshly prepared in DMSO. To determine the effect of ginsenoside Rg3 in EPCs, preliminary experiments were done to determine the effective dose of Rg3 in EPC. As a result, a significant different dose of Rg3 in EPC was identified (Rg3 60, 300 ng/mL).

Isolation of $CD34^+$ cells and *ex vivo* expansion of $CD34^+$ cells. Human cord blood (HUCB) was supplied by Pusan National University Hospital. The $CD34^+$ cells were isolated from human cord blood as described previously (Suuronen *et al*., 2006). Total MNCs were isolated from cord blood by Ficoll-gradient density centrifugation. The $CD34^+$ 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. Next, 2×10^4 $CD34^+$ cells were expanded using EPC expansion culture methods recently established in our laboratory (Kwon *et al*., 2009). The harvested enriched $CD34^+$ cells were cultured in serum-free medium (StemSpan™; StemCell Technologies, Vancouver, BC, Canada) 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-3 L) for 7 days, after which floating cells were collected. The number of expanded cells was counted by a hemocytometer. In order to address the phenotype of surface markers expressed on CD34 cells expanded with ginsenoside Rg3, fluorescence-activated cell sorting (FACS) analysis was carried out with a FACSCalibur analyser (Becton Dickinson, San Jose, CA) and CellQuest™ Pro software (Becton Dickinson Immunocytometry Systems, Mountain View, CA). These cells were stained with FITC-conjugated anti-CD34 (BD Pharmingen, San Diego, CA), phycoerythrin (PE)-conjugated anti-CD133 (Miltenyi Biotec), phycoerythrin (PE)-conjugated anti-CD31 (BD Pharmingen), and phycoerythrin (PE)-conjugated anti-KDR (BD Pharmingen) antibodies.

EPC colony forming assay. Human CD34(+) cells were cultured in the methyl cellulose medium (for human cells) MethoCult (R) SF H4236 (Stemcell Technologies) with 20 ng/mL stem cell derived factor (SCF), 50 ng/mL vascular endothelial growth factor (VEGF), 20 ng/mL interleukin-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 18 days. The cell density was 1.5×10^3 cells/dish. The EPC-CFUs were identified as large EPC-CFUs or small EPC-CFUs by visual inspection with an inverted microscope under $\times 40$ magnification. Large EPC-CFUs were composed of spindle-shaped cells and small EPC-CFUs were composed of round adhesive cells.

EPC-CFU staining. After 18 days in culture, and washing in methylcellulose-containing medium with PBS, the EPC-CFU cultures were treated with 0.4 μg/mL 1,1′-dioctadecyl-3,3,3′,3-tetramethyl-indocarbocyanine perchlorate-labeled acLDL (acLDL-Dil; Biomedical Technologies Inc., Stoughton, MA) for 1 h, and fixed in 1 mL of 4% paraformaldehyde (PFA) for 30 min at room temperature. After washing twice with PBS, the cultures were reacted with fluorescein isothiocyanate (FITC)-conjugated CD31 (BD Pharmingen) overnight at 4 °C. After washing twice with PBS, the cultures were stained with DAPI for 30 min at room temperature. After washing with PBS, the cultures were observed under a fluorescence microscope.

To check whether colony-derived cells have endothelial characteristics, flow cytometry analysis was performed using fluorescein isothiocyanate (FITC)-conjugated CD31 antibodies (BD Pharmingen) as well as the uptake capacity of 1,1′-dioctadecyl-3,3,3′,3-tetramethyl-indocarbocyanine perchlorate-labeled acLDL (acLDL-Dil; Biomedical Technologies Inc., Stoughton, MA). Briefly, the colony-derived cells were incubated with acLDL-Dil for 5 h and further stained for CD31 antibodies. The stained cells were analysed by FACS flow cytometry (Becton Dickinson) and Cell Quest Software counting 10000 events per sample.

Western blot analysis. Proteins were separated by SDS-PAGE and transferred to a membrane (Immobilon-P; Millipore). The membranes were blocked in blocking solution (Tris-buffered saline, TBS) containing 0.1% (v/v) Tween-20 and 5% (v/v) non-fat skim milk for 1 h and then incubated overnight with anti-phospho-Akt-Ser^{473} (1:2000), anti-Akt (1:1000), anti-phospho-eNOS-Ser^{1177} (1:1000) and anti-eNOS (1:1000) antibodies (Cell Signaling Technology), or anti-VEGF (1:1000), anti-SDF-1 (1:1000), anti-FGF-2 (1:1000) and anti-β-actin (1:1000) antibodies (Santa Cruz Biotechnology). The membranes were washed extensively in Tris-buffered

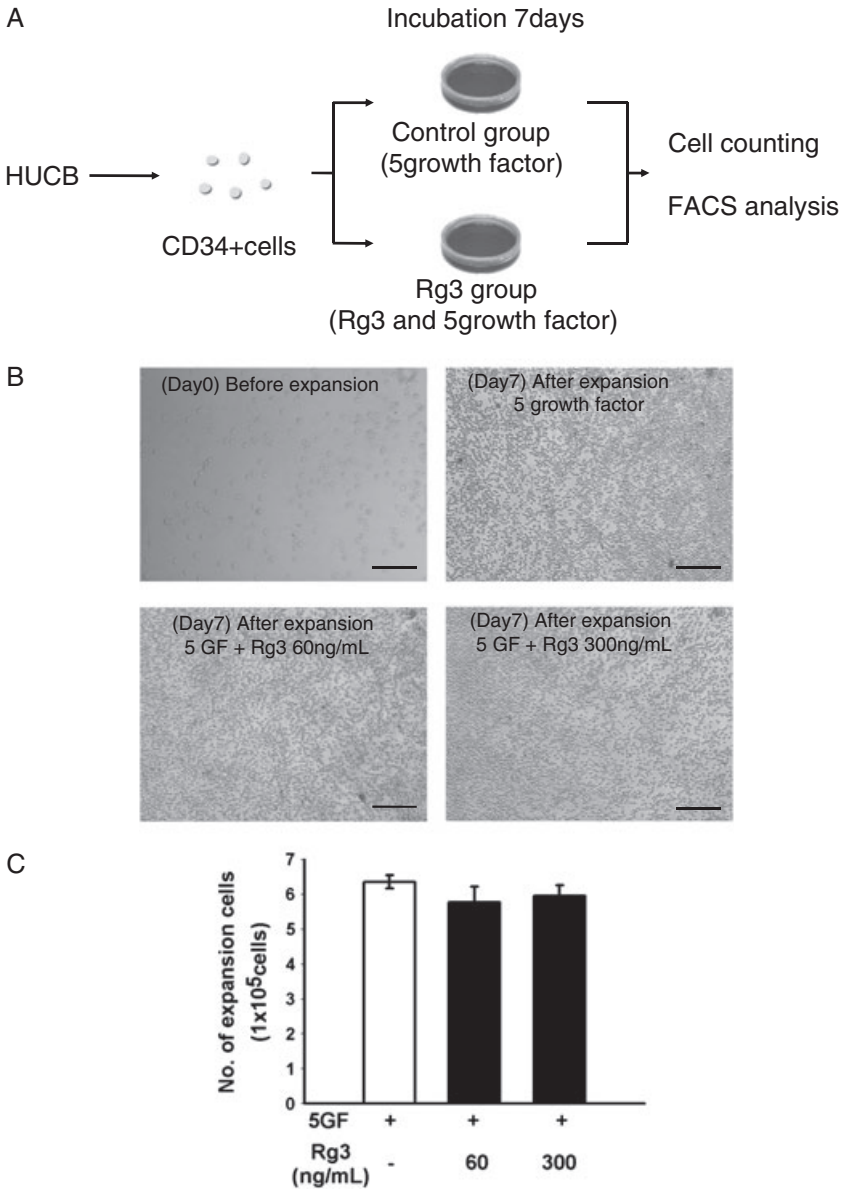

Figure 1. Effect of ginsenoside Rg3 on *ex vivo* expansion of $CD34^+$ cells. (A) Outline of $CD34^+$ cell expansion experiment. (B) Morphology of $CD34^+$ cells before and after expansion. The round shaped cells were observed in all groups. Scale bar = 250 μm. (C) The number of $CD34^+$ cells before and after expansion. There was no significant difference between the groups (n = 5).

saline containing 0.1% (v/v) Tween-20 before incubation with a secondary anti-mouse (1:1000) or anti-rabbit (1:3000) antibody conjugated to horseradish peroxidase for 1 h. After washing with PBS, protein was then visualized using ECL solution (GE Healthcare, Buckinghamshire, UK) and x-ray film.

Wound healing assay. *Ex vivo* cultured EPCs (1.5×10^5 cells/well) were starved with EBM-2 medium containing 0.1% FBS for 24 h. For the wound healing assays, the monolayers grown on 24-well plates were wounded with a micropipette tip. The cells were washed to remove the debris, incubated for 8 h in low-serum media containing different ginsenoside Rg3 concentrations and 100 ng/mL VEGF, and observed by optical microscopy.

Tube formation assay. *Ex vivo* cultured EPCs (2×10^4 cells/well) were seeded on a 96-well culture plate coated with 55 μL per well of matrigel matrix (BD, 10 mL vial, Bedford, MA) for 30 min at 37 °C. The plates were examined for tube formation after 6 h incubation. After incubation, the level of tubular formation was determined, after randomly taking a picture per well at × 40 magnification by optical microscopy. The tube length was measured using image-J software (http://rsb.info.nih.gov).

Statistical analysis. A statistical comparison of the two groups was performed using a Student's t-test. These results were also analysed statistically using the Statview 5.0 software package (Abacus Concepts, Inc., Berkeley, CA). Scheffé's test was performed for multiple comparisons between each group after ANOVA. All data, which were obtained from at least three independent experiments, are expressed as mean ± standard deviation. A value of $p = 0.05$ or less was considered statistically significant.

RESULTS

Effect of ginsenoside Rg3 on *ex vivo* expansion of CD34$^+$ cells

To determine the effect of ginsenoside Rg3 on a serum-free culture of stem cells, cord blood-derived CD34$^+$ cells were isolated and expanded as indicated in Materials and Methods (Fig. 1A). The cell number expanded from 2×10^4 to $6.35 \times 10^5 \pm 0.19 \times 10^5$. Rg3 activated CD34$^+$ cells expanded to $5.77 \times 10^5 \pm 0.45 \times 10^5$ and $5.95 \times 10^5 \pm 0.30 \times 10^5$. As shown in Fig. 1B and C, there was no significant difference in the number of expanded cells or the morphological changes (Fig. 1B and C), which suggested that ginsenoside Rg3 did not affect stem cell proliferation.

Effect of ginsenoside Rg3 on surface markers on CD34$^+$ cells

FACS analysis revealed changes in endothelial lineage markers, including CD31 and KDR, as well as several progenitor surface markers, including CD34 and CD133, in expanded cells. However, as shown in Fig. 2A, there was no significant difference in the expression of several surface markers on expanded CD34 cells, regardless of the presence of Rg3. To accurately identify the number of surface markers, an absolute value was calculated. As a result, Rg3 (300 ng/mL) significantly reduced the expression of CD34 and KDR after stem cell expansion, although treatment with Rg3 did not affect the number of CD133 and CD31 positive cells (Fig. 2B).

Effect of ginsenoside Rg3 on bioactivities of CD34-derived EPCs

To determine the effect of ginsenoside Rg3 on the bioactivities of CD34-derived EPCs, multiple EPC functional assays were performed including a wound healing assay and a tube formation assay. As shown in Supplementary Fig. 1A and 1B, ginsenoside Rg3 significantly inhibited the VEGF dependent migratory

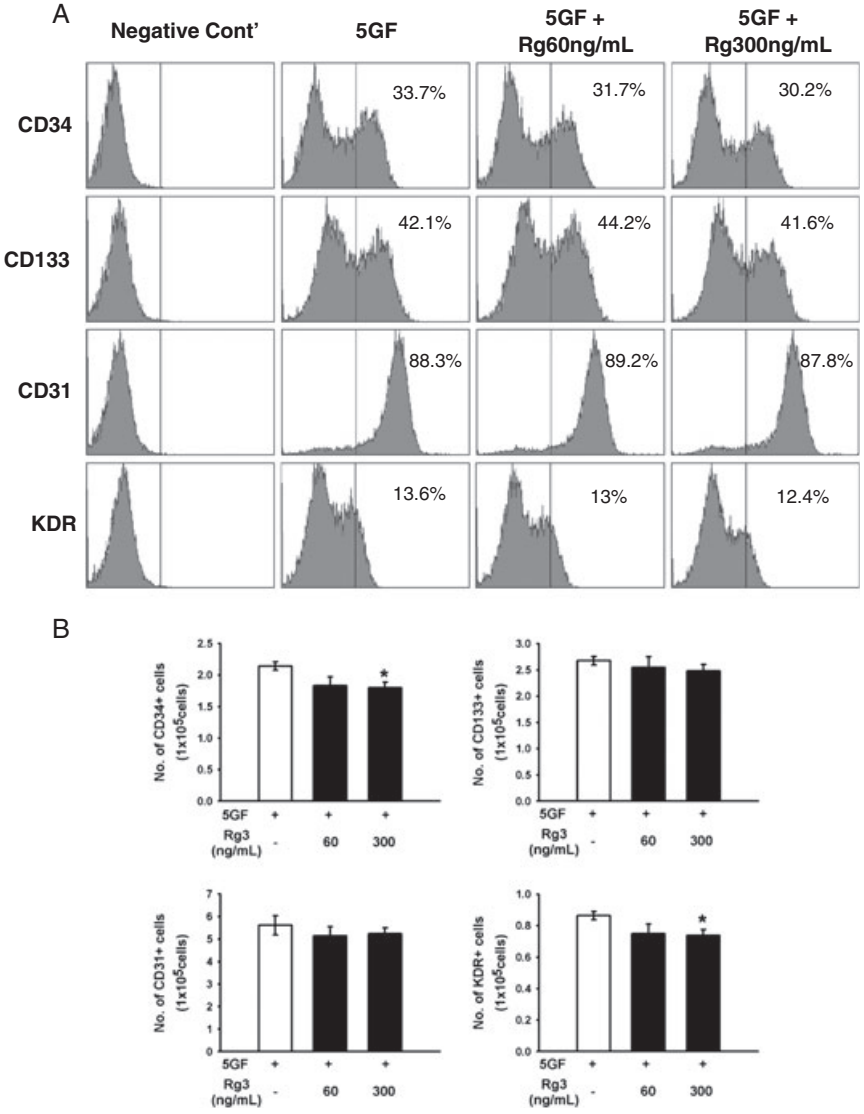

Figure 2. Effect of ginsenoside Rg3 on alteration of surface markers in CD34$^+$ cells. (A) Alteration of CD34$^+$ cell surface makers after treatment with either 5 growth factors alone or 5 growth factors and Rg3 for 7 days. (B) Absolute changes in cell surface markers on expanded CD34$^+$ cells. The number of CD34$^+$ and KDR$^+$ cells was significantly decreased in the Rg3-treated group ($n = 5$) $*p < 0.05$, $**p < 0.005$.

capability of EPCs dose-dependently. Moreover, it was demonstrated that ginsenoside Rg3 also markedly inhibited tubular forming capacity of EPCs (Supplementary Fig. 1C and 1D). To evaluate the effect of ginsenoside Rg3 on the secretion of angiogenic cytokines, western blot analysis was performed using anti-VEGF, anti-SDF-1 and anti-FGF-2 antibodies. As shown in Supplementary Fig. 1E, ginsenoside Rg3 did not affect cytokine production activities.

Effect of ginsenoside Rg3 in the EPC colony-forming assay (EPC-CFA)

To determine whether ginsenoside Rg3 inhibits the differentiation of human cord blood-derived $CD34^+$ stem cells into EPCs, the EPC-CFA was performed. After the $CD34^+$ cells were isolated from human cord blood, the cells were cultured in a previously developed differentiation culture medium for 18 days (Fig. 3A). After 18 days, two types of colonies were observed. Large colonies were composed of large spindle-shaped cells, whereas small colonies contained mainly small round cells. The endothelial characteristics of the cells in the colonies were examined after uptake of Dil-conjugated Ac-LDL (Dil-Ac-LDL) and chemical binding with FITC-conjugated CD31, a standard marker of endothelial lineage cells. The EPCs were identified as double positive cells by fluorescence microscopy (Fig. 3B) and flow cytometry analysis (Fig. 3C). The EPC-CFUs were significantly decreased in the Rg3-treated groups. In the case of small colonies, the average number of CFUs decreased from 16.66 ± 0.12 (control groups) to 7.66 ± 0.27 and 7.66 ± 0.07 (Rg3-treated groups). Rg3 inhibited the small colony-forming ability by more than 50% (Fig. 4A). In contrast, the average number of large CFUs was 2 in the control groups and 2.33 ± 0.24 and 1.33 ± 0.86 in the Rg3-treated groups

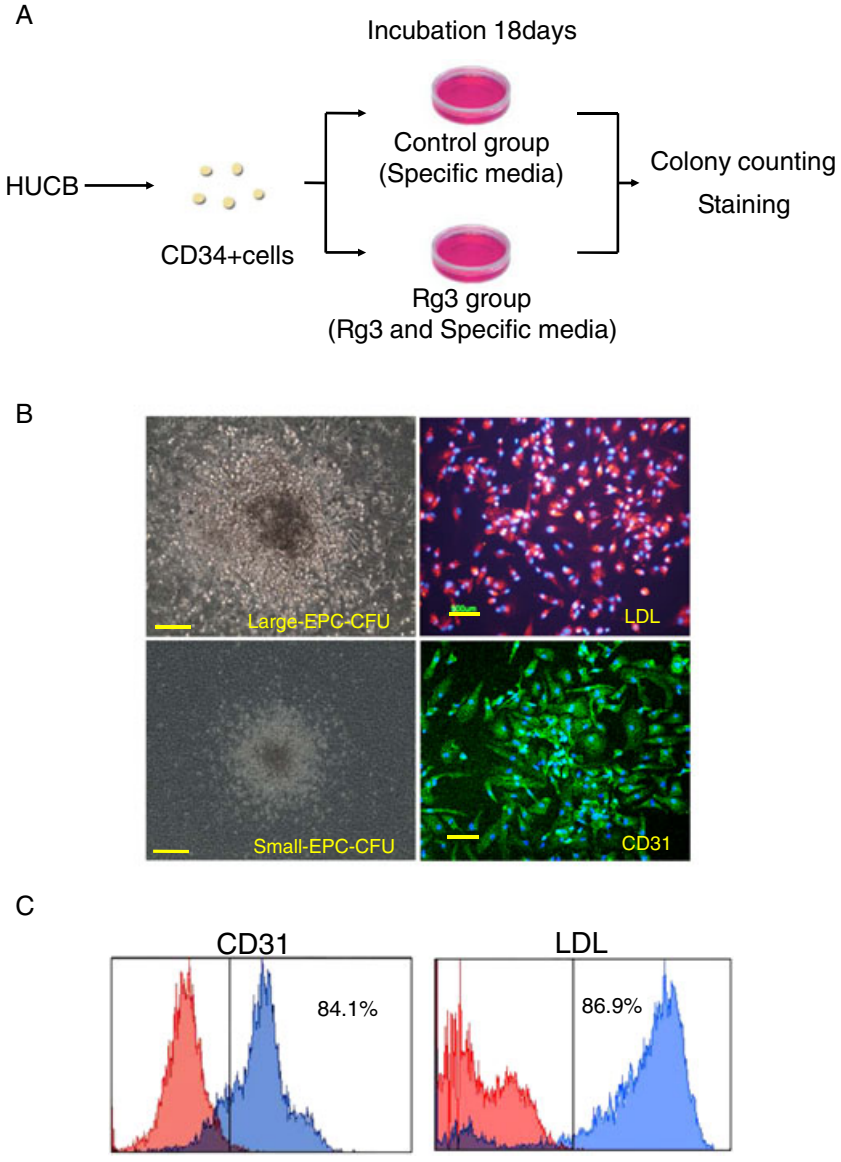

Figure 3. Colony forming assay. (A) Experimental scheme for the EPC-colony forming assay (CFA). (B) Representative images of the two types of EPC colony-forming units. In large colonies, the cells are relatively large and spindle shaped. Small colonies are composed of relatively small cells that are round shaped. EPC-CFUs were identified as double positive cells due to acLDL-Dil uptake (red) and CD31 reactivity (green), a typical endothelial markers. (C) Flow cytometry analysis of colony-derived cells. EPC-CFUs dominantly expressed the endothelial marker, CD31 surface marker, as well as acLDL-Dil uptaking capacity. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

(Fig. 4B). Therefore, there was no significant difference in the number of large CFUs. The number of total CFUs was significantly decreased from 18.66 ± 0.11 (control groups) to 9.99 ± 0.17 and 8.99 ± 0.11 (Rg3-treated groups) (Fig. 4C). As a result, the data indicate that Rg3 had inhibitory effects on EPC differentiation, especially on the formation of small EPC colonies, an early differentiation stage in EPC commitment.

Effect of ginsenoside Rg3 on the p-Akt and p-eNOS pathway in CD34-derived EPCs

Western blot analysis was performed using anti-p-Akt, anti-Akt, anti-p-eNOS and anti-eNOS antibodies using Rg3-treated or untreated CD34-derived EPCs. β-Actin was probed as a protein loading control. Consistent with the result obtained by Feliers *et al.* (2005) and Namiecinska *et al.* (2005), VEGF activated Akt (phospho-Akt) and eNOS (phospho-eNOS). Importantly, treatment with ginsenoside Rg3 significantly inhibited the VEGF-induced activation of Akt and eNOS

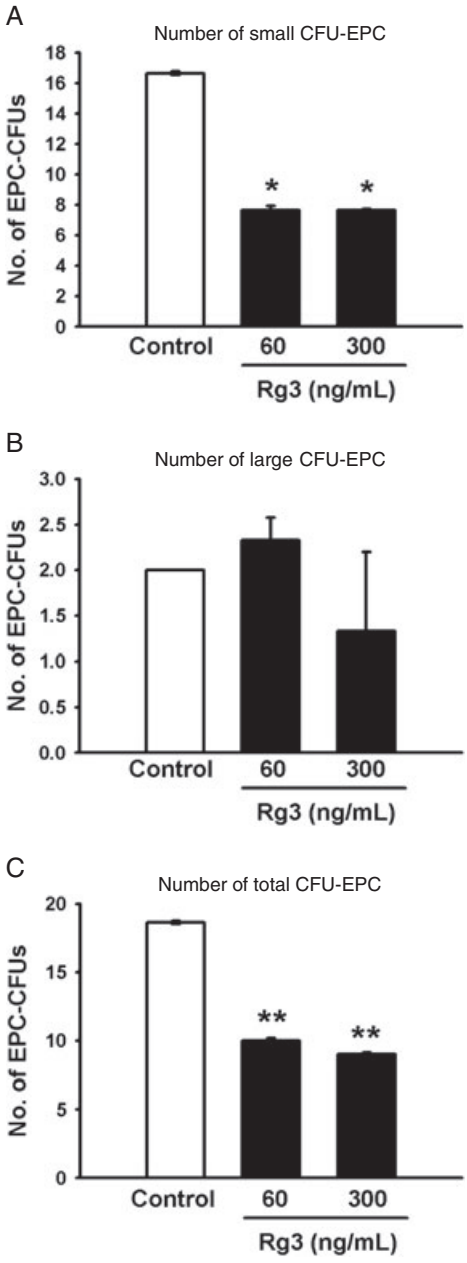

Figure 4. Effect of ginsenoside Rg3 on EPC-CFA. (A) Graph of small EPC-CFUs. Small colony forming units were significantly decreased in the Rg3-treated group, and colony formation in the Rg3-treated group was inhibited by more than 50% compared with that in the control group. (B) Graph of large EPC-CFUs. In contrast, there was no significant inhibitory effect of Rg3 on large colony forming units. (C) Graph of total EPC-CFUs. The total colony number was significantly decreased in the Rg3-treated group ($n = 3$).

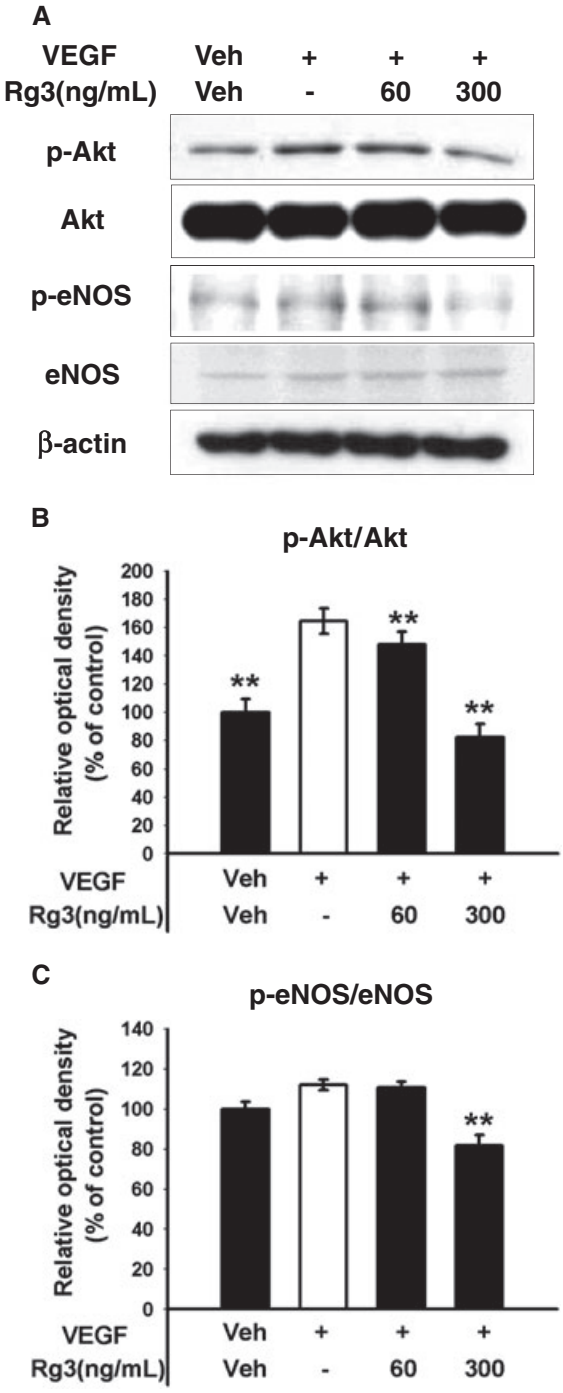

Figure 5. Ginsenoside Rg3 inhibited the VEGF-dependent Akt/eNOS pathway in CD34-derived EPCs. (A) Treatment with ginsenoside Rg3 attenuated phospho-Akt and phospho-eNOS protein levels in CD34-derived EPCs. Total Akt, total eNOS and β-actin were probed as protein loading controls. (B) The graph shows the relative optical density of phospho-Akt/Akt. The expression of p-Akt was significantly decreased in Rg3-treated groups. (C) The graph shows the relative optical density of phospho-eNOS/eNOS. The expression of p-eNOS was significantly decreased in the Rg3-treated group (300 ng/mL) ($n = 3$) $^{*}p < 0.05$, $^{**}p < 0.005$.

(Fig. 5A, 5B and 5C), indicating that ginsenoside Rg3 inhibits EPC differentiation, in part via inhibition of VEGF dependent Akt/eNOS signaling.

DISCUSSION

A recent study showed that EPCs could be classified into primitive and definitive EPCs. Primitive EPCs contained more immature cells derived from hematopoietic stem cells, which subsequently differentiate into definitive EPCs. In the CFA assay, small EPC-CFUs have primitive EPC characteristics and a strong potential for proliferation. Furthermore, small EPCs give rise to secondary large EPC colonies when reseeded. On the other hand, large EPC-CFUs have definitive EPCs characteristics. Large EPCs also have a predominantly vasculogenic potential, including cell adhesion and tube-like structure formation *in vitro*, as well as high *in vivo* activity toward *de novo* blood vessel formation after transplantation into a murine ischemic hind limb model. Large EPCs did not form secondary colonies when reseeded, but instead gave rise to isolated endothelial lineage cells (Masuda *et al*., 2012). To investigate the effect of ginsenoside Rg3 on the commitment of CD34 stem cells into EPCs, EPC colony-forming assays were performed with freshly isolated $CD34^+$ cells from HUCB. It was shown that ginsenoside Rg3 significantly attenuated EPC differentiation of HUCB-derived CD34-positive stem cells. In particular, it reduced the number of small EPC-CFUs, suggesting that an early step in EPC differentiation is more affected by ginsenoside Rg3 than later steps in differentiation. Small EPCs had higher proliferative capacity than large EPCs, and secreted vasculogenic cytokines, including vascular endothelial growth factor (VEGF), stromal cell derived factor (SDF), fibroblast growth factor (FGF) and angiopoietin-1. Therefore, the data suggest that ginsenoside Rg3 controls the commitment steps to the EPC lineage of HUCB-derived CD34-positive stem cells rather than the maturation steps leading to functional EPCs.

The present data showed that ginsenoside Rg3 did not affect cell proliferation or the morphology of EPCs during CD34 expansion; however, as demonstrated by flow cytometry analysis, it reduced the expression of EPC surface markers, including CD34 and KDR (VEGFR2). Likewise, our recent data from western blotting analysis indicate that ginsenoside Rg3 inhibits VEGFR2 (KDR) expression (unpublished observation). Mechanistically, Rg3 might inhibit the transcription of VEGFR2 or its activation through either the regulation of VEGFR2 phosphorylation or by directly blocking VEGF binding to receptor 2. Emerging evidence has shown that VEGF-induced activation of Akt increases the phosphorylation of eNOS, and as a result, NO production increased in EPCs (Fulton *et al*., 1999; Gerber *et al*., 1998). Based on the present data and previous reports, which imply that eNOS-derived NO can promote differentiation of EPCs, it is reasonable to conclude that EPC differentiation might be inhibited by treatment with ginsenoside Rg3 through inhibition of the Akt/eNOS axis.

In conclusion, this study showed that ginsenoside Rg3 inhibited EPC differentiation, in part, via abrogating the VEGF-dependent Akt/eNOS signaling pathways in EPCs as well as inhibition of EPC bioactivities including migration and tube formation. Since the contribution of EPCs during tumor angiogenesis is pivotal in the initiation and promotion of tumor neovessel formation through the production of angiogenic cytokines (Lyden *et al*., 2001; Nolan *et al*., 2007), these results provide some evidence for ginsenoside Rg3 as a potential drug candidate for the control of angiogenesis. In the future, further experiments investigating the underlying molecular mechanism of ginsenoside Rg3 in EPC biology will help determine the roles of EPC bioactivities in the control of the angiogenic switch during tumor angiogenesis.

Acknowledgements

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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