Ginsenoside Rd regulates the Akt/mTOR/p70S6K signaling cascade and suppresses angiogenesis and breast tumor growth

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Abstract. Blockade of angiogenesis is an important approach for cancer treatment and prevention. In the present study, we investigated the effect of ginsenoside Rd (Rd) on angiogenesis *in vitro* and *in vivo*. Our results demonstrated that Rd inhibited vascular endothelial growth factor (VEGF)-induced migration, tube formation and proliferation of primary cultured human umbilical vascular endothelial cells (HUVECs) dose-dependently. Furthermore, Rd abrogated VEGF-induced sprouting of the vessels from aortic rings, and inhibited vascular formation in the Matrigel plug assay *in vivo*. Under normoxic or hypoxic conditions, Rd suppressed VEGF-induced activation of Akt/mammalian target of rapamycin (mTOR) signaling transduction cascades in HUVECs. When intraperitoneally administered to mice bearing human breast cancer (MDA-MB-231) cell xenografts, Rd significantly decreased the volume and the weight of solid tumors in a dose-dependent manner, and decreased tumor angiogenesis as less Ki67- and CD31-positive cells were found. Additionally, we found that Rd inhibited proliferation and induced apoptosis as well as the inhibition of Akt/mTOR/P70S6 kinase signaling in breast cancer cells. Collectively, our findings revealed that Rd may be a promising anti-angiogenic drug with significant antitumor activity in human breast cancer.

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Abbreviations: HUVECs, human umbilical vascular endothelial cells; VEGF, vascular endothelial growth factor; HIF-1α, hypoxia-inducible factor-1α; mTOR, mammalian target of rapamycin; ECGS, endothelial cell growth supplement

Key words: ginsenoside Rd, tumor angiogenesis, Akt/mTOR/p70S6K, HIF-1α/VEGF, breast cancer

Introduction

Angiogenesis, a process involving the formation of new blood vessels from pre-existing vessels, is an essential event in a variety of physiological processes such as embryonic development, ovulation and wound healing, as well as pathological conditions such as cancer, chronic inflammation, arthritis, aneurysms and arteriovenous malformations (1,2). It is now well-known that angiogenesis is vital for tumor growth, invasion and metastasis, which contribute to over 90% of deaths in various types of cancers, including human breast cancer (3,4). Modulating tumor-associated angiogenesis thus represents a promising strategy for the development of anticancer therapies (5,6). In the last decades, several drugs that target tumor vascularization and inhibit tumor angiogenesis have been developed and approved by the US Food and Drug Administration for clinical use, such as the humanized anti-VEGF-A antibody bevacizumab, and the tyrosine kinase inhibitors sorafenib and sunitinib (6,7).

The vascular endothelial growth factor (VEGF) family of proteins play a pivotal role in tumor angiogenesis by increasing vascular permeability and endothelial cell proliferation, migration and invasion into surrounding tissues (8). Cellular responses to VEGF are mainly mediated by the receptor tyrosine kinase VEGFR2 (also known as Flk-1) on the surface of endothelial cells (9). The activation of Akt/mTOR/p70S6K mediated by the HIF-1α/VEGF-receptor (VEGFR) alliance triggers many functions in tumorigenesis such as tumor cell proliferation, angiogenesis and metastasis (10-12). Consequently, the discovery of novel HIF-1α/VEGF and Akt/mTOR/p70S6K pathway inhibitors shows great promise for anticancer therapeutics.

Panax ginseng (*P. ginseng*) is a traditional herbal medicine popular in China, Korea and Japan. It has a wide range of beneficial effects in the treatment of cardiovascular or cerebrovascular diseases, immune deficiency, aging, as well as cancer (13,14). Saponins, commonly known as ginsenosides, are the main active ingredients in *P. ginseng*. Among more than 150 ginsenosides that have been identified (15), ginsenoside Rd (Rd) (Fig. 1A) has attracted increasing attention. It displays a remarkable neuroprotective effect on cerebral ischemia (16), and can attenuate myocardial ischemia-reperfusion

injury (17). Moreover, increasing evidence indicates that Rd exerts significant antiproliferative/pro-apoptotic effects on diverse cancers, including breast, gastric, liver and cervical cancers (18-20), through the negative regulation of various oncogenic molecules such as the melastatin type transient receptor potential 7 (TRPM7) channel, cell cycle progression or the induction of caspase activity. However, there is no evidence on its anti-angiogenic potential and effect on the Akt/mTOR/p70S6K signaling cascade. In the present study, we reported for the first time that Rd suppressed VEGF-induced angiogenesis and the Akt/mTOR/p70S6K signaling cascade under both normoxic and hypoxic conditions in human umbilical vascular endothelial cells (HUVECs), an extensively used *in vitro* model for angiogenesis research (21). Our findings may contribute to the potential use of Rd as an anticancer drug.

Materials and methods

Reagents. Rd was obtained from the Shanghai Research Center for Standardization of Chinese Medicines (Shanghai, China). Its structure was confirmed using ^{1}HNMR and ^{13}C NMR spectral analysis, and its purity was >98% as determined by high pressure liquid chromatography (HPLC) analysis.

Phospho-p85 PI3K (Tyr458), PI3K, phospho-Akt (Thr308), Akt, phospho-mTOR (Ser2481), mTOR, cleaved caspase-3, Bax, Bcl-2, VEGFR2, GAPDH, goat anti-rabbit horseradish peroxidase (HRP)-conjugated, and goat anti-mouse HRP antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Other antibodies against Ki67, HIF-1α and CD31 were provided by Abcam (Cambridge, UK). The Pierce BCA protein assay kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Human recombinant VEGF was supplied by PeproTech (Rocky Hill, NJ, USA). All of the other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Cell lines. HUVECs were cultured in endothelial cell medium (ECM; ScienCell, San Diego, CA, USA) supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (both from Gibco, Gaithersburg, MD, USA). Breast cancer cell line MDA-MB-231 obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 100 U/ml of penicillin and 100 μg/ml streptomycin. All the cells were cultured at 37˚C with 95% humidity and a 5% CO_2 gas environment.

Cell viability assay. HUVECs or MDA-MB-231 cells were treated with or without VEGF (10 ng/ml) and Rd for 48 h. The cell viability was determined using MTT assay (Sigma-Aldrich). The number of cells were counted after trypsinizing HUVECs. In addition, the final cell viability and the numbers of the treated cells were expressed as a percentage relative to that of the untreated control cells.

Flow cytometric analysis. MDA-MB-231 cells were treated with Rd at 0, 25 and 50 μM for 24 h. The cells were collected by centrifugation at 400 x g, and stained with propidium iodide (PI) (50 μg/ml) and Annexin V-FITC (2 μg/ml) for 15 min in the dark. The staining was then immediately analyzed by flow cytometry using the FACScan and CellQuest program. The FCS Express program (BD Biosciences, San Jose, CA, USA) was used to determine the percentage of apoptotic cells.

Wound healing migration assay. The wound healing migration assay was performed as previously described (22). Briefly, HUVECs were treated with mitomycin C to inactivate cell proliferation. Scratches were drawn with sterile pipette tips. Fresh ECM was added with or without VEGF (10 ng/ml) and different concentrations of Rd. Images of the cells were captured using an inverted microscope (Olympus CKX41; Olympus, Tokyo, Japan) after incubation at 37˚C for 10 h. The width of the scratches was evaluated and used as the indicator for the assessment of cell migration ability.

Capillary-like tube formation assay. After incubation with ECM containing 1% FBS for 4 h, HUVECs were seeded at a density of $1x10^4$ cells/well into Matrigel (BD Biosciences, Bedford, MA, USA) coated 96-well plates followed by treatment with Rd at different concentrations for 4 h. Tubes forming intact networks were quantified by counting the number of branch points from 5 random fields/well in a blinded manner under an inverted microscope.

Rat aortic ring assay. Rat aortic ring assay was performed as previously described (23). In brief, aortas isolated from Sprague-Dawley rats were cleaned of fibroadipose tissue and collateral vessels, and cut into rings of 1-1.5 mm of thickness. The aortic rings were randomly placed into growth factor reduced Matrigel-coated 48-well plates and further overlayed with 100 μl of Matrigel. Medium with or without VEGF (10 ng/ml) supplemented with different concentrations of Rd was added to the wells and incubated with the aortic rings for 6 days. At the end of the incubation period, the microvessel sprouts that had formed were fixed and photographed using an inverted microscope. After images were acquired, the outgrowth area was delineated and measured using Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA), and used for the assessment of angiogenesis.

Matrigel plug assay. Matrigel plug assay is a widely used method to assess the *in vivo* anti-angiogenic effect of drugs (24). To examine the anti-angiogenic property of Rd, Matrigel (0.5 ml) containing 100 ng VEGF and 20 U of heparin with or without Rd (25 and 50 μM) were subcutaneously injected into the ventral area of female C57BL/6 mice (5 weeks old, n=6/group). After 7 days, the mice were sacrificed and the intact Matrigel plugs were isolated and photographed. The hemoglobin in the Matrigel plugs was quantified using Drabkin's reagent kit (Sigma-Aldrich) according to the manufacturer's instructions. The concentration of hemoglobin was calculated based on a set of hemoglobin standards. Blood vessels in the Matrigel were visualized with an antibody against CD31.

Xenograft mouse model. Healthy 5-week-old female athymic nude mice (BALB/c) were obtained from Shanghai Laboratory Animal Center. All studies were performed in accordance with the guidelines approved by the Animal Ethics Committee of Shanghai University of TCM (SHUTCM). MDA-MB-231

cells were subcutaneously injected ($1x10^7$ cells/mouse) into the right flank of each mouse. Treatments were started 4 days after tumor cell implantation and lasted for 4 weeks. After the tumors grew to ~50 mm^3, the tumor-bearing mice were randomly assigned into 5 groups (n=10/group): the vehicle control, the doxorubicin (DOC; 10 mg/kg, once a week for 4 weeks), and the Rd groups (1, 3 and 10 mg/kg). The vehicle control group received the vehicle solvent [0.1% v/v dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS)]. The Rd groups were intraperitoneally administered with Rd diluted in vehicle solvent daily. The body weight of the mice was monitored once a week. The tumors were assessed every day using a digital caliper. The tumor volume was calculated using the formula: V (mm^3) = [ab^2] x 0.5, where a is the length, and b is the width of the tumor. At the end of treatment, the mice were sacrificed and the tumors of the mice from the different groups were collected for further analysis.

Immunohistochemical analysis. Solid tumors were fixed with 10% phosphate-buffered formalin, embedded in paraffin and longitudinally sectioned at 5-μm of thickness. The sections were incubated with 3% H_2O_2 for 10 min to deactivate the endogenous peroxidase. For antigen retrieval, the sections were soaked in 10 mM citrate buffer solution (pH 6.0), and heated twice in the microwave oven. The slides were then washed thoroughly with PBS (pH 7.4). After being blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich) in TBS for 20 min, the sections were incubated with primary antibodies against CD31 and Ki67 at 4˚C overnight followed by a thorough wash with PBS. Afterwards, the slides were sequentially incubated with a biotinylated secondary antibody for 20 min and streptavidin-HRP for another 20 min. The staining was visualized after incubation with a DAB-H_2O_2 solution. The slides were then counterstained with hematoxylin for 1 min, dehydrated with ethanol and sealed in resin for microscopic observation.

Western blot analysis. Cell and tissue homogenates were lysed in lysis buffer containing 50 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 20 mM NaF, 0.5% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin A on ice. After centrifugation at 12,000 x g for 15 min at 4˚C, the supernatant was collected and the protein concentration was determined using the BCA method. Total proteins, 30 μg for each sample, were separated on 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Blocking was performed in 5% BSA (Sigma-Aldrich) in 0.1% Tween-20 in PBS (PBST) for 1 h. The membranes were probed with respective primary antibodies overnight at 4˚C. Binding of the primary antibody was detected using a peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA) for 1 h at room temperature. The blots were developed using ECL detection reagents (GE Healthcare, Waukesha, WI, USA). The gray intensity of the protein bands was quantified using ImageJ and normalized to that of GAPDH in each sample.

Statistical analysis. To examine the difference among multiple groups, one-way ANOVA followed by Tukey's multiple comparison test were conducted with GraphPad Prism 5.0. The unpaired t-test was used to assess the difference between two groups. All data are presented as the mean ± SEM. A value of $p<0.05$ was considered as a significant difference.

Results

Rd inhibits VEGF-induced migration, vascularization and viability of HUVECs. As endothelial cell migration is one of the most important and early events during the process of angiogenesis (25), the wound-healing migration assay was performed to determine the effects of Rd on HUVEC migration. Upon stimulation with VEGF, HUVECs migrated much faster and the wounds healed faster compared with the control (Fig. 1B; $p<0.01$). Rd treatment at 25 and 50 μM significantly prevented VEGF-induced migration of HUVECs as the wound healing was delayed compared to the VEGF-treated cells ($p<0.01$ and $p<0.001$). Tube formation assay represents a simple, reliable and powerful model for studying inhibitors of angiogenesis. As shown in Fig. 1C, cells stimulated with VEGF formed robust tubular structures when seeded on growth factor-reduced two-dimensional Matrigel ($p<0.05$). The addition of Rd suppressed the formation of the capillary-like network ($p<0.05$ and $p<0.01$). The process of angiogenesis also requires the proliferation of endothelial cells. VEGF alone promoted the cell viability and increased the number of HUVECs (Fig. 1D; $p<0.05$, $p<0.01$). Rd treatment (5, 10, 25 and 50 μM) mitigated the VEGF-induced cell viability and number of HUVECs in a dose-dependent manner ($p<0.05$, $p<0.01$ and $p<0.001$). Overall, these findings clearly demonstrated that Rd exerted an anti-angiogenic effect through the inhibition of cell proliferation, migration and tube formation of endothelial cells.

Rd mitigates VEGF-induced angiogenesis ex vivo and in vivo. To study whether Rd affected VEGF-induced angiogenesis *ex vivo*, an aortic ring assay was conducted. As shown in Fig. 2A, VEGF treatment significantly stimulated microvessel sprouting, leading to the formation of a network of vessels around the aortic rings ($p<0.001$). The addition of Rd at 25 and 50 μm significantly counteracted the VEGF-induced microvessel sprouting which appeared to be achieved in a dose-dependent manner ($p<0.05$ and $p<0.01$).

To further verify the inhibitory effect of Rd on angiogenesis *in vivo*, the Matrigel plug assay was carried out. As shown in Fig. 2B, Matrigel plugs containing VEGF alone appeared reddish-brown, inside of which increased hemoglobin was found ($p<0.001$), indicating the formation of functional vasculatures. Accordingly, more CD31 immunoreactive capillaries were found within the VEGF-treated Matrigel plugs (Fig. 2C) and the capillary density was significantly higher ($p<0.001$), compared with the vehicle-treated control. In contrast, Rd at 25 and 50 μM markedly inhibited VEGF-induced hemoglobin accumulation in the Matrigel plugs as the color of the Rd-treated Matrigel plugs became bleached (Fig. 2B; both $p<0.001$). Meanwhile, CD31 immunoreactive capillaries were decreased in Rd-treated Matrigel plugs (Fig. 2C; $p<0.001$). All of these results demonstrated that Rd effectively inhibited angiogenesis *in vivo*.

Rd inhibits the VEGF-mediated signaling cascade for angiogenesis. Interaction of VEGFR2 with VEGF leads to

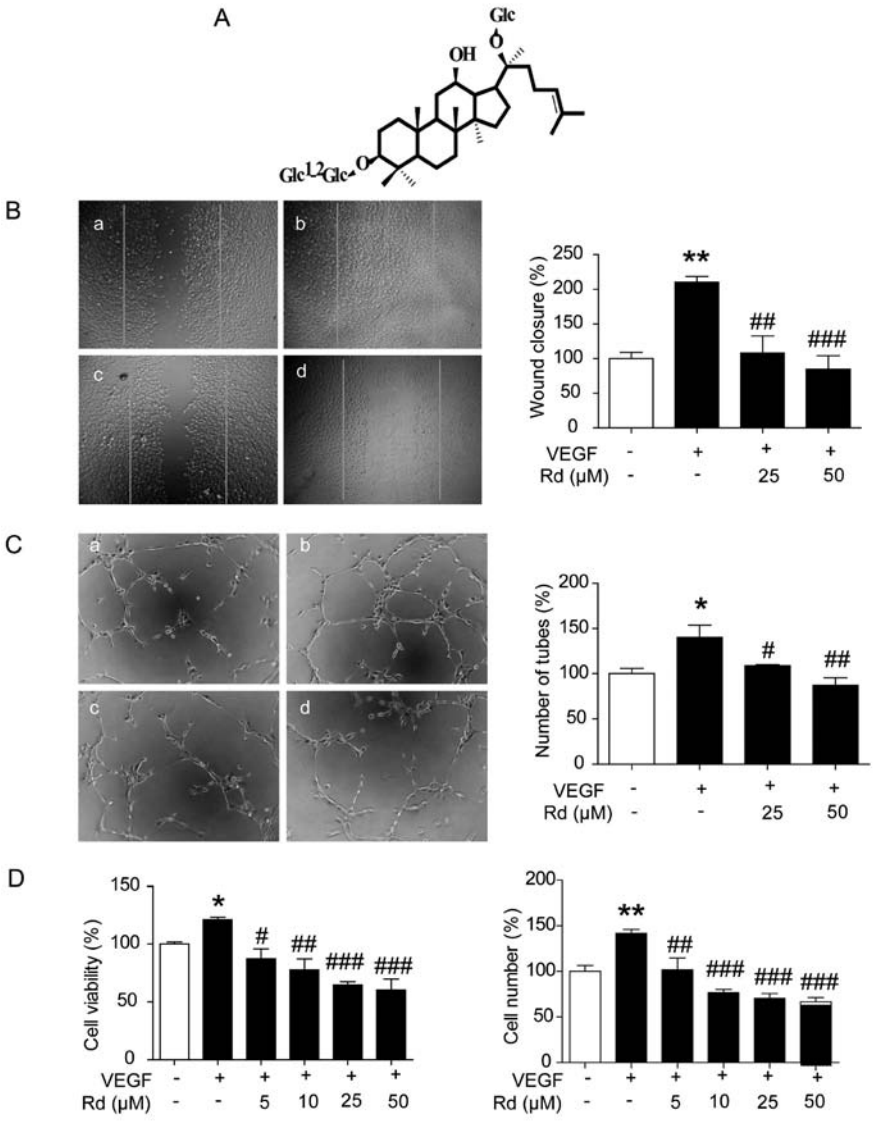

Figure 1. Rd inhibits the VEGF-induced proliferation, migration and capillary-structure formation of HUVECs. (A) The molecular structure of ginsenoside Rd. (B) Rd inhibited HUVEC migration. HUVECs were scratched by pipette tips and treated with or without VEGF (10 ng/ml) and Rd. The migrated cells were quantified by assessing the width of the scratches and are expressed as the percentage to that of the untreated cells. a, control; b, VEGF; c, VEGF + Rd (25 μM); d, VEGF + Rd (50 μM). (C) Rd inhibited VEGF-induced tube formation in Matrigel. Tubes forming intact networks were quantified by counting the number of branch points from 5 random fields/well in a blinded manner under an inverted microscope. a, control; b, VEGF; c, VEGF + Rd (25 μM); d, VEGF + Rd (50 μM). (D) Rd inhibited VEGF-induced cell viability and the number of HUVECs; *P<0.05, **P<0.01 vs. the control; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. VEGF alone. Rd, ginsenoside Rd; HUVECs, human umbilical vascular endothelial cells; VEGF, vascular endothelial growth factor.

the activation of various downstream signaling molecules responsible for endothelial cell migration, proliferation and survival (26). HIF-1α is a key regulatory protein in hypoxic response, which is downstream of mTOR signaling and is an important modulator of VEGF (27). To further elucidate the underlying mechanism of the anti-angiogenic effect of Rd, the activation of the signaling molecules in HUVECs were examined under both normoxic and hypoxic conditions.

As shown in Fig. 3A, under normoxic conditions, VEGF induced the expression of VEGFR2, thereby, enhancing the phosphorylation of the PI3K/Akt/mTOR signaling molecules. As a result, downstream p70S6K and HIF-1α that are crucial to the regulation of protein synthesis and angiogenesis (28) were also phosphorylated. Conversely, VEGF-induced VEGFR2 was suppressed by Rd in a dose-dependent manner. Meanwhile, the PI3K/Akt/mTOR signaling pathway molecules as well as p70S6K and HIF-1α activated by VEGF were all inhibited with Rd treatment. We next examined the effect of Rd on the VEGF signaling cascade under hypoxic conditions using $CoCl_2$, a reagent used widely for the induction of hypoxia (29,30). Not surprisingly, $CoCl_2$ treatment enhanced the activation of the PI3K/Akt/mTOR/p70S6K signals and increased the expression of HIF-1α and VEGFR2 (Fig. 3B). However, similar to its effect under normoxic conditions, Rd treatment diminished the angiogenic signals induced by $CoCl_2$ on HUVECs. Therefore, Rd inhibited the VEGF-mediated PI3K/Akt/mTOR/p70S6K signaling cascade activation in both normoxic and hypoxic conditions.

Rd inhibits tumor growth and tumor angiogenesis in a xenograft mouse model. To investigate the effect of Rd on tumor growth and tumor angiogenesis *in vivo*, a human breast

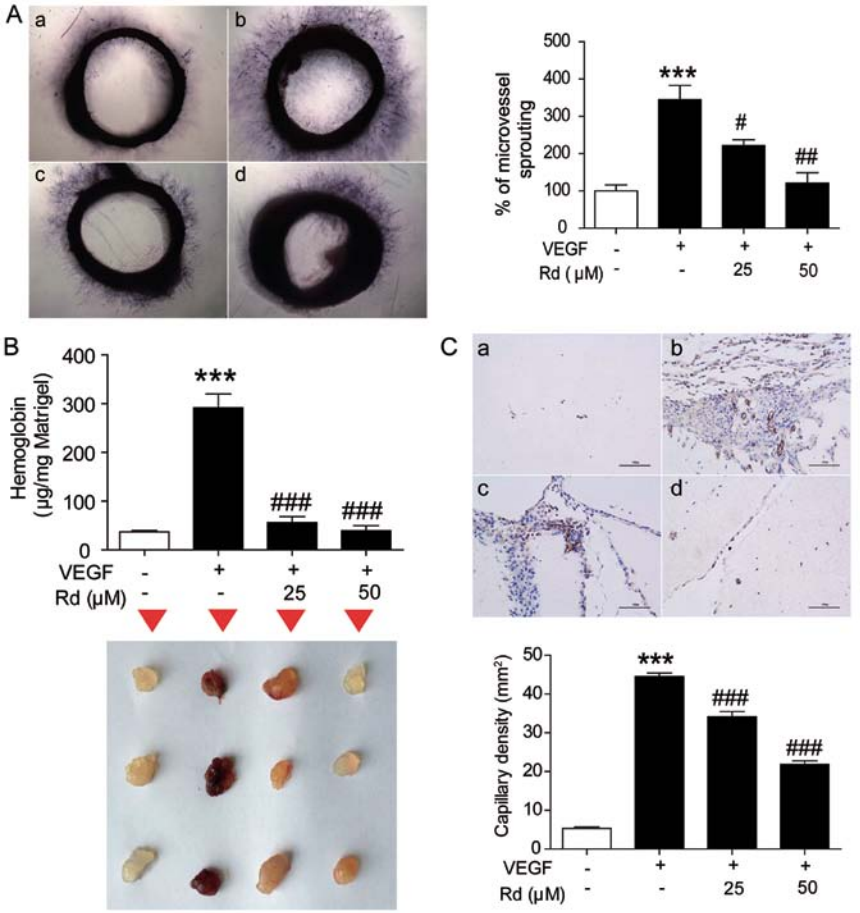

Figure 2. Rd mitigates VEGF-induced angiogenesis *ex vivo* and *in vivo*. Aortic segments isolated from Sprague-Dawley rats were placed in the Matrigel-covered wells and treated with VEGF (10 ng/ml) in the presence or absence of Rd. (A) Representative images and the average microvessel area of sprouts from the margins of aortic rings (n=4/group). a, control; b, VEGF; c, VEGF + Rd (25 μM); d, VEGF + Rd (50 μM). (B) Upper panel, the hemoglobin content of Matrigel plugs from the indicated groups (n=3/group). Lower panel, the representative images of the Matrigel plugs from the indicated groups. (C) Rd inhibited blood vessel formation in Matrigel plugs. The Matrigel plugs were fixed, sectioned and stained with the anti-CD31 antibody (n=3/group). Upper panel, immunostaining of CD31. Scale bar, 100 μm. Lower panel, CD31 positive capillary density; ***P<0.001 vs. the control; $^{\#}$P<0.05; $^{\#\#}$P<0.01; $^{\#\#\#}$P<0.001 vs. VEGF alone. Rd, ginsenoside Rd; VEGF, vascular endothelial growth factor.

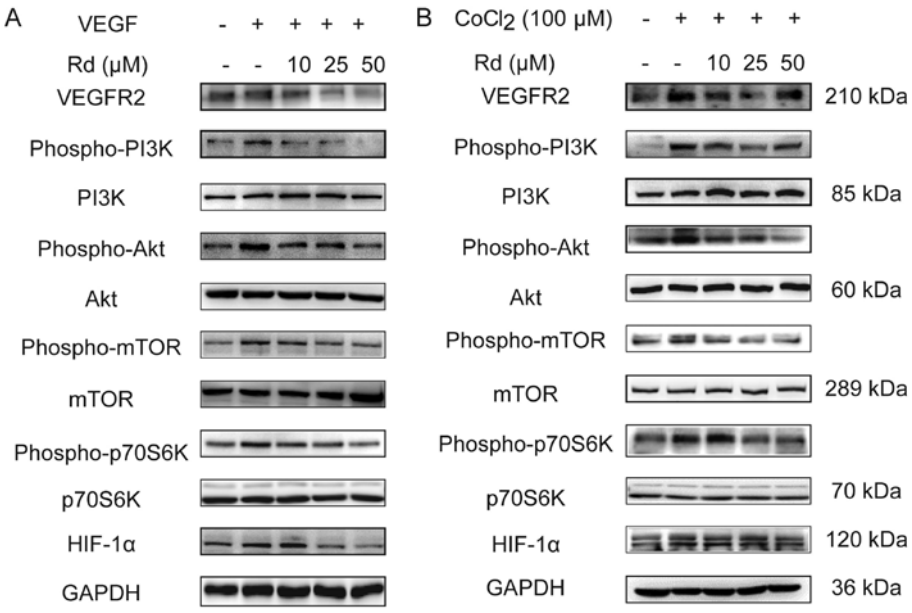

Figure 3. Rd modulates the expression of VEGFR2 and activation of the PI3K/Akt/mTOR pathway in HUVECs under normoxic or hypoxic conditions. (A) HUVEC cell lysates were prepared after 48 h of treatment with Rd at indicated concentrations and specific antibodies were used for the detection of the indicated proteins. (B) Hypoxia was induced with cobalt chloride ($CoCl_2$; 100 μM) and cells were treated with Rd for 48 h. Cells were lysed as described in Materials and methods, and immunoblotted with the indicated antibodies. Rd, ginsenoside Rd; HUVECs, human umbilical vascular endothelial cells.

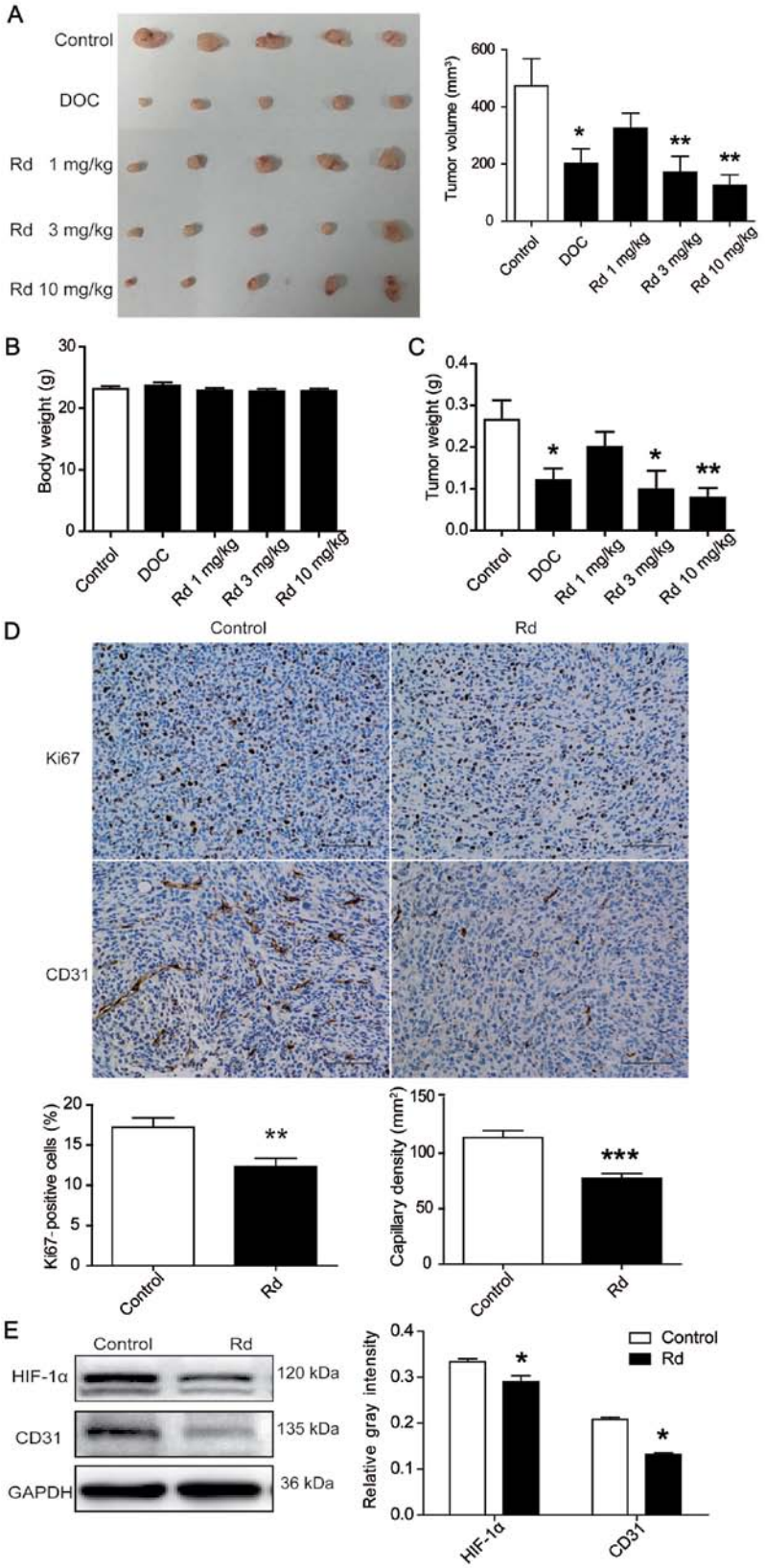

Figure 4. Rd prevents tumor growth and tumor angiogenesis in xenografted mice. MDA-MB-231 cells were injected into 6-week-old BALB/c nude mice ($1x10^7$ cells/mouse). After solid tumors grew to ~50 mm^3, the mice were subcutaneously injected with or without Rd (1, 3 and 10 mg/kg/day) or DOC (10 mg/kg, once a week for 4 weeks). (A and C) The tumor volume and weight change after Rd administration. (B) The body weight change of mice treated with Rd. (D) Immunohistochemical analysis of Ki67 and CD31 of Rd-treated (3 mg/kg/day) tumors. Scale bar, 100 μm. (E) Western blot analysis of HIF-1α and CD31 in Rd-treated (3 mg/kg/day) tumors (n=5/group); $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. the control. Rd, ginsenoside Rd; DOC, doxorubicin.

tumor-bearing xenograft mouse model was employed. As shown in Fig. 4A, the administration of Rd at 3 and 10 mg/kg for 28 days substantially suppressed the tumor volume (Fig. 4A; $p<0.01$) and decreased the tumor weight (Fig. 4C; $p<0.05$ or

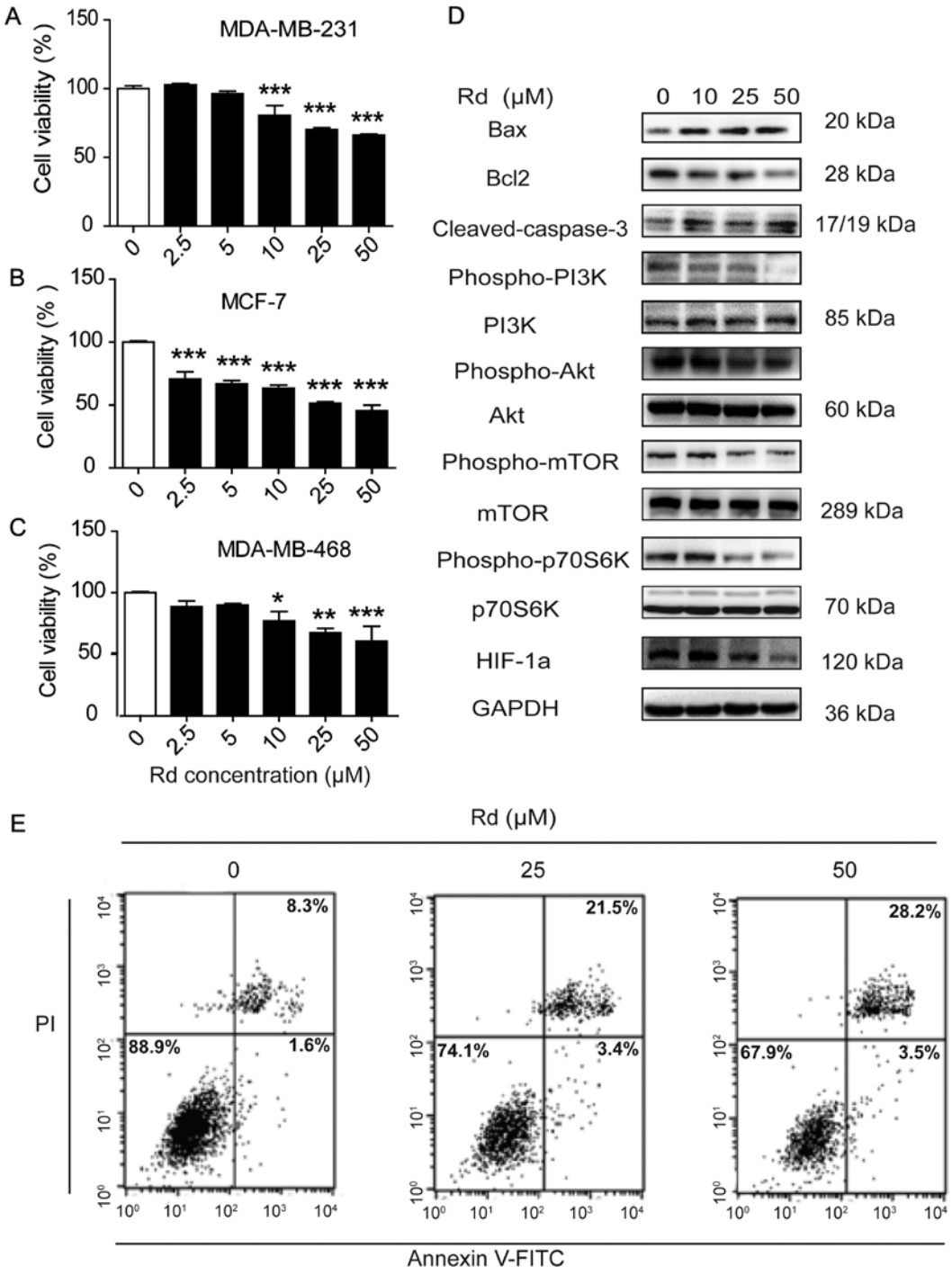

Figure 5. Rd induces cell apoptosis and modulates the Akt/mTOR/P70S6K pathway in breast cancer cells. (A-C) Rd inhibited cell viability of MDA-MB-231, MCF-7 and MDA-MB-468 cancer cells in a dose-dependent manner. The cell viability was quantified by MTT assay. (D) Western blot analysis of Bax, Bcl-2, cleaved caspase-3 and the phosphorylation of the mTOR signaling pathway molecules in Rd-treated MDA-MB-231 cells. (E) Flow cytometric analysis of apoptosis after Annexin V/PI staining. Rd, ginsenoside Rd.

$p<0.01$ in a dose-dependent manner. Notably, administration of Rd at all experimental doses exhibited no obvious toxicity on solid tumor model animals as no significant loss of body weight occurred during the course of the experiment (Fig. 4B). We next evaluated the effect of Rd on cell proliferation and angiogenesis in the solid tumors by immunohistochemical analysis. As shown in Fig. 4D, the number of Ki67 (a marker of cell proliferation) immunoreactive cells in tumor tissues of Rd-treated (3 mg/kg) mice was less than that in the control group of mice ($p<0.01$). Moreover, CD31 immunoreactive capillaries were decreased in the Rd-treated tumors ($p<0.001$). In addition, Rd treatment led to a decrease in the expression of HIF-1α and CD31 protein (Fig. 4E; $p<0.05$). All of these results revealed that Rd prevented angiogenesis and tumor growth in mice.

Rd induces apoptosis in breast cancer cells. Since Rd effectively decreased cell proliferation in the xenografted breast tumors, we next examined whether it also had a direct influence on breast cancer cells. As shown in Fig. 5A-C, Rd treatment dose-

dependently decreased the cell viability of cancer cell lines, such as MDA-MB-231, MCF-7 and MDA-MB-468. Flow cytometric analysis revealed that Rd significantly increased the percentage of the apoptotic cells (Fig. 5D). Furthermore, Rd treatment increased Bax and cleaved caspase-3 while it decreased Bcl-2 expression in MDA-MB-231 cells, implicating its pro-apoptotic effect. Rd treatment also inhibited the activation of PI3K, Akt, mTOR and p70S6K and mitigated the expression of HIF1-α in MDA-MB-231 cells (Fig. 5E). All of these results indicated that Rd induced direct apoptosis of breast cancer cells, which might be mediated through the inhibition of PI3K/Akt/mTOR signaling pathway.

Discussion

The process of angiogenesis plays a crucial role in cancer progression as the newly formed tumor vasculature serves initially as feeding tubes providing nutrients and oxygen supply for the growing tumor mass, and finally as conduits for dissemination of tumor cells that escape from the established primary tumor (31). The current strategies in anticancer therapy become ineffective once tumor cells reach favored secondary organs and generate metastatic foci. Therefore, control of tumor angiogenesis has become a central issue in the fight against cancer progression (32). In the present study, we demonstrated that ginsenoside Rd (Rd), a potent angiogenic inhibitor, prevented angiogenesis through multiple steps, including endothelial cell viability, migration and differentiation into capillary-like structures. In addition, it modulated the Akt/mTOR/p70S6K signaling pathway in a relatively specific manner both in endothelial cells and in breast cancer cells, leading to its overall anti-breast tumor effect in tumor-bearing mice.

Proliferation, migration and formation of tubular structures of endothelial cells are indicators for the development of new blood vessels from the pre-existing vascular bed in angiogenesis (33,34). As VEGF is the major mediator of tumor-associated angiogenesis, we investigated the effect of Rd on angiogenesis in different *in vitro* and *in vivo* models upon VEGF stimulation. In HUVECs, Rd effectively abrogated VEGF-induced migration, invasion and capillary-like structure formation. Furthermore aortic ring capillary formation and Matrigel plug assays confirmed the anti-angiogenetic effect of Rd. In mice bearing breast tumors, Rd administration was also found to inhibit CD31-positive capillary formation. All of our results demonstrated that Rd exerted a robust anti-angiogenic function.

VEGF exerts its biological effects by binding to transmembrane receptors such as VEGFR1 and VEGFR2, both of which are specifically expressed on the surface of endothelial cells and contain a cytoplasmic tyrosine kinase domain (35). Therapies targeting the VEGF-receptor have been demonstrated to inhibit angiogenesis and tumor growth in preclinical models (36-38). Therefore, the VEGF/VEGFR pathway has become a major focus of basic research and drug development for cancer therapy. In the present study, Rd substantially downregulated the VEGF-induced activation of VEGFR2 in HUVECs, thereby indicating that the anti-angiogenic effects of Rd may be partially mediated through the inhibition of VEGFR2 activation.

The PI3K/Akt/mTOR pathway is involved in the regulation of multiple cellular processes, including cell proliferation, migration, invasion and survival. In numerous types of cancers this pathway is overactive, decreasing apoptosis, allowing proliferation, and thus, enhanced signaling through this pathway is a significant contributor to new blood vessel formation (39,40). Activation of p70S6K, the kinase downstream of mTOR, frequently leads to the activation of HIFs which regulate tumorigenesis, angiogenesis and tumor growth through VEGF (27,41). In the present study, treatment with Rd substantially inhibited proliferation of HUVECs and cancer cells, and decreased the activation of Akt/mTOR/p70S6K as well as HIF-1α in both endothelial and breast cancer cells, suggesting the important role of the pathway in the anticancer effect of Rd.

Induction of apoptosis of tumor cells is one of the characteristics of most anticancer drugs. Apoptosis can be triggered by various stimuli through either extrinsic or intrinsic pathways. Generally, the extrinsic pathway includes the signaling transduction from death receptors and caspase-3 while the intrinsic pathway involves mitochondrial apoptotic proteins Bcl-2, cytochrome *c* and Bax (42). In the present study, Rd treatment modulated the expression of Bcl-2, Bax and caspase-3, and increased the percentage of apoptotic cells in MDA-MB-231 cells, suggesting the regulatory effect of Rd on both the extrinsic and intrinsic pathways of apoptosis.

In conclusion, our results demonstrated that Rd administration inhibited angiogenesis both *in vitro* and *in vivo*, which may be mediated via the inhibition of HIF-1α/VEGF through the Akt/mTOR/p70S6K signaling pathway. Our novel findings may facilitate the potential application of Rd against breast cancer.

Acknowledgements

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