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Matairesinol inhibits angiogenesis via suppression of mitochondrial reactive oxygen species

Boram Lee, Ki Hyun Kim, Hye Jin Jung, Ho Jeong Kwon *

Chemical Genomics National Research Laboratory, Department of Biotechnology, Translational Research Center for Protein Function Control, College of Life Science & Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea

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ABSTRACT

Mitochondrial reactive oxygen species (mROS) are involved in cancer initiation and progression and function as signaling molecules in many aspects of hypoxia and growth factor-mediated signaling. Here we report that matairesinol, a natural small molecule identified from the cell-based screening of 200 natural plants, suppresses mROS generation resulting in anti-angiogenic activity. A non-toxic concentration of matairesinol inhibited the proliferation of human umbilical vein endothelial cells. The compound also suppressed *in vitro* angiogenesis of tube formation and chemoinvasion, as well as *in vivo* angiogenesis of the chorioallantoic membrane at non-toxic doses. Furthermore, matairesinol decreased hypoxia-inducible factor-1α in hypoxic HeLa cells. These results demonstrate that matairesinol could function as a novel angiogenesis inhibitor by suppressing mROS signaling.

1. Introduction

Angiogenesis is the process of new blood vessel formation that is dependent upon oxygen and nutrient concentrations [1]. Low-oxygen tumor microenvironments trigger hypoxia-induced tumor angiogenesis that promotes tumor cell survival and metastasis. Mitochondrial reactive oxygen species (mROS) have been implicated as one of the key players in angiogenic hypoxic signaling [2]. However, the detailed mechanisms and factors of mROS generation and subsequent hypoxic signaling remain to be elucidated.

Natural product-derived small molecules have been used as molecular probes in biology and as therapeutic agents for preventing and curing a number of diseases, including cancer. For instance, paclitaxel, an active component of *Pacific yew*, binds to and stabilizes microtubules, which leads to cell cycle arrest. Because of this unique biological activity of paclitaxel, the compound has been used as a valuable probe for investigating microtubular cytoskeleton organization [3]. Moreover, paclitaxel is one of the most popular drugs for treating a number of cancers including breast and lung cancer and Kaposi's sarcoma [4,5]. Given the great benefits of natural small molecules, there is increasing interest in identifying new, natural small molecules with unique chemical structures and biological activities.

As part of our continuous efforts to discover new anti-angiogenic agents in natural plants by using cell-based screening, we screened 200 crude extracts of natural Nepalese plants for their effects on suppressing mROS generation under hypoxia. We discovered that matairesinol [(αR,βR)-α,β-bis(4-hydroxy-3-methoxybenzyl)butyrolactone, Fig. 1A] suppresses mROS and possesses anti-angiogenic properties. An active principle of *Cedrus deodara* (Roxb.) G. Don, matairesinol is a lignan precursor for mammalian lignans [6]. The plant is a species of cedar native to western Nepal. Its crude extract is used for antifungal and anticancer properties [7]. However, the detailed mechanism by which this naturally occurring small molecule exerts its anticancer activity has not been explored. Here, we report for the first time that matairesinol is a novel natural, small molecule that inhibits angiogenesis capable of suppressing mROS generation at a nontoxic dose.

2. Materials and methods

2.1. Materials

Matairesinol was purchased from Sigma–Aldrich (Sigma–Aldrich, Saint Louis, MO). Endothelial basal media-2 (EBM-2) was purchased from Cambrex Bio Science (Walkersville, MD). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), MitoSOX (red mitochondrial superoxide indicator) and Hoechst 33342 were purchased from Invitrogen (Grand Island, NY). Transwell plates, recombinant human vascular endothelial cell growth factor (VEGF), and Matrigel were obtained from Corning

Abbreviations: Ang 2, angiopoietin 2; CAM, chorioallantoic membrane; mROS, mitochondrial reactive oxygen species; RA, retinoic acid; VEGF, vascular endothelial growth factor.

* Corresponding author. Fax: +82 2 362 7265.
E-mail address: kwonhj@yonsei.ac.kr (H.J. Kwon).

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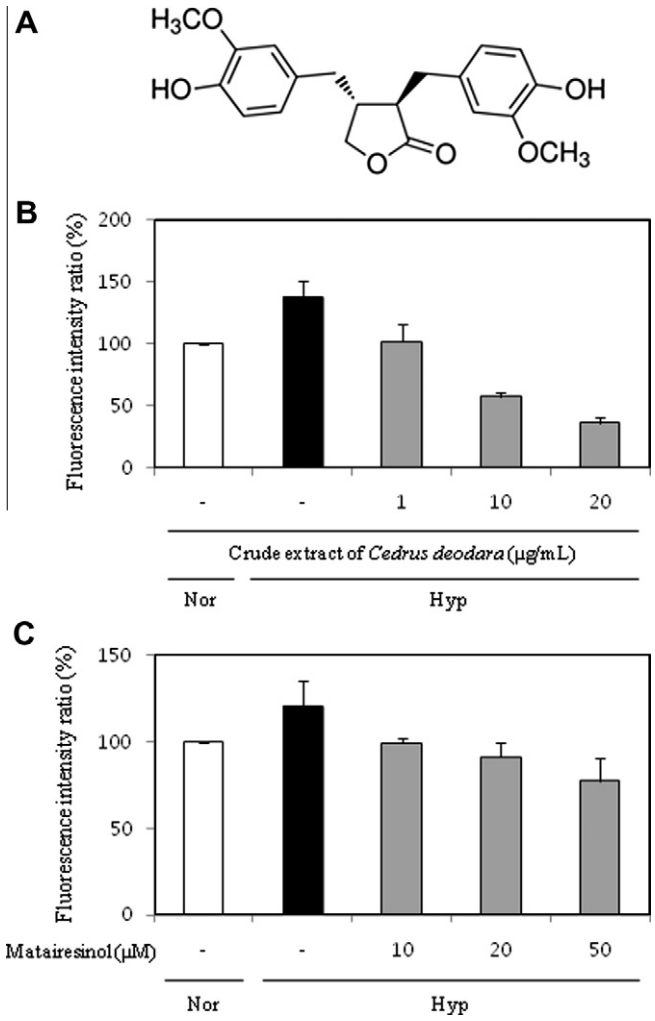

Fig. 1. Chemical structure and mitochondrial reactive oxygen species (mROS) suppressive activity of matairesinol in HeLa cells. (A) The chemical structure of matairesinol ($C_{20}H_{22}O_6$, MW 358.39). (B) The effect of crude extract of *Cedrus deodara* (Roxb.) G. Don on mROS generation during hypoxia. (C) The effect of matairesinol on mROS generation under hypoxia.

(Cambridge, MA), KOMA Biotech., Inc. (Seoul, Korea), and BD Biosciences (Bedford, MA), respectively. Anti-hypoxia-inducible factor-1α (HIF-1α), anti-cyclinD1 and anti-tubulin antibodies were purchased from BD Biosciences (Bedford, MA), Cell Signaling (Beverly, MA), and Millipore (Billerica, MA), respectively.

2.2. Cell culture and hypoxic conditions

Human umbilical vascular endothelial cells (HUVECs) were grown for 4–10 passages in EBM-2 medium supplemented with 10% FBS. HeLa (human cervical carcinoma) cells were grown in DMEM with 10% FBS and 1% antibiotics. Both cell lines were maintained at 37 °C under a humidified atmosphere of 5% CO_2 incubator. For hypoxic conditions, cells were incubated at 5% CO_2 with 1% O_2 balanced with N_2 in an anaerobic chamber (Forma).

2.3. Cell growth and viability assay

HUVECs were seeded onto 96-well plates, incubated for 24 h, and treated with varying concentrations of compounds for 72 h. Cell growth was measured using a 3-(4,5-dimehylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma–Aldrich) colorimetric assay, and viability assay was assessed using trypan blue staining [8]. Cellular morphology was observed with an Olympus IX70 microscope at 100× magnification (Olympus America, Inc., Melville, NY).

2.4. Measurement of mROS levels

mROS levels were assayed with MitoSOX. After incubation with MitoSOX (5 μM) and Hoechst 33342 for 10 min, the cells were washed once with washing buffer and fixed with 4% formaldehyde. Cell images and data were automatically obtained using the ArrayScan V^{TI} HCS Reader (Cellomics, Inc., Pittsburgh, PA) [9]. For these experiments, the compartmental Analysis of BioApplication was to identify individual cells by their Hoechst-labeled nuclei and then automatically analyzed MitoSOX fluorescence intensity changes with each cell.

2.5. In vitro capillary tube formation assay

Matrigel (10 mg/mL) was used to coat 48-well culture plates and allowed to polymerize for 2 h at 37 °C. HUVECs (7×10^4 cells/well) were seeded on the matrigel surface and treated with VEGF (30 ng/mL) [10,11]. Small molecules were added for 3–16 h at 37 °C. Cellular morphological changes and tube formations were observed under a microscope (IX71, Olympus) and photographed (DP70, Olympus).

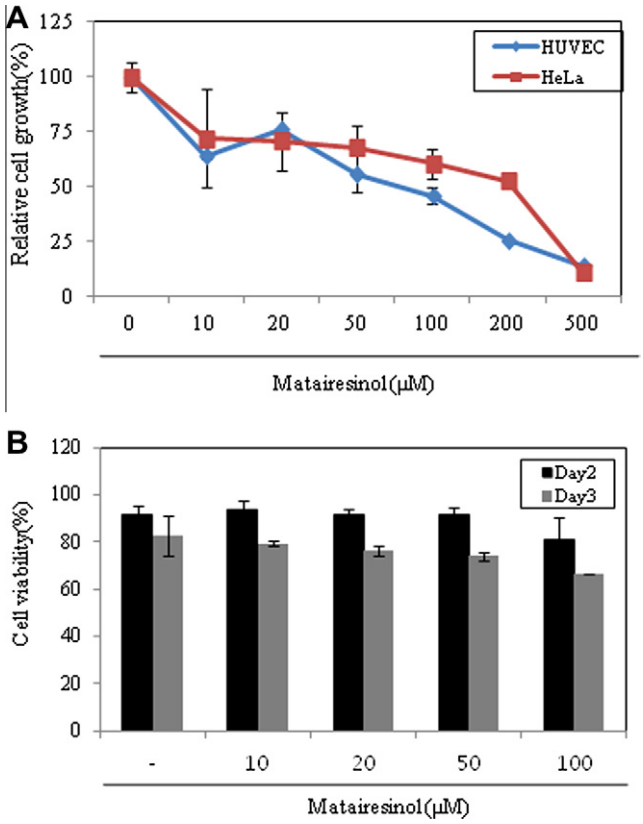

Fig. 2. Anti-proliferative activity of matairesinol on human umbilical vein endothelial cells (HUVECs) and HeLa cells. (A) The effect of matairesinol on cell proliferation. HUVECs and HeLa cells were treated with matairesinol for 3 days, and cell growth was measured using the 3-(4,5-dimehylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. (B) The effect of matairesinol on cell viability as determined by the trypan blue assay.

2.6. In vitro chemoinvasion assay

HUVEC invasiveness was examined *in vitro* using a Transwell chamber system with 8.0-μm-pore-size polycarbonate filter inserts [12]. The bottom of the filter was coated with 10 μL of gelatin (1 mg/mL), and the top was coated with 10 μL of Matrigel (3 mg/mL). Matairesinol were added to the lower chamber in the presence of VEGF (30 ng/mL), and HUVECs (7×10^5 cells/well) were placed in the top of the filter. The chamber was incubated at 37 °C for 18 h. The cells were fixed with 70% methanol and stained with hematoxylin and eosin, and their invasiveness was determined by counting the total number of cells in the lower side of the filter with an Olympus IX70 microscope at 100× magnification.

2.7. Chorioallantoic membrane (CAM) assay

In vivo anti-angiogenic activity was conducted using the CAM assay, as previously described [13]. Fertilized chicken eggs were kept in a humidified incubator at 37 °C for 3 days. Approximately 4–5 mL of egg albumin was removed with a hypodermic needle after allowing the CAM and yolk sac to drop away from the shell membrane. On day 5, the shell membrane was peeled away and a compound-loaded Thermanox coverslip (NUNC, Rochester, NY) were applied to the CAM surface. Two days later, 1 mL of Intralipose (Greencross Co., Korea) was injected beneath the CAM, and the membrane was observed under a microscope. Retinoic acid (RA), a well-known anti-angiogenic compound, served as a positive control.

2.8. Measurement of VEGF by enzyme-linked immunosorbent assay

The VEGF concentration in media from matairesinol-treated cells was determined using a VEGF Immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The results were expressed as concentration of VEGF relative to the total amount of VEGF in each well [14].

2.9. Western blot analysis

The cell lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) using standard electroblotting procedures. Blots were then blocked and immunolabeled overnight at 4 °C with primary antibodies, including anti-HIF-1α, anti-cyclinD1, and anti-tubulin antibodies. Immunolabeling was detected by an enhanced chemiluminescence kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions.

2.10. Statistical analysis

Results are expressed as the mean ± standard error. Student's *t*-tests were used to determine statistical significance between

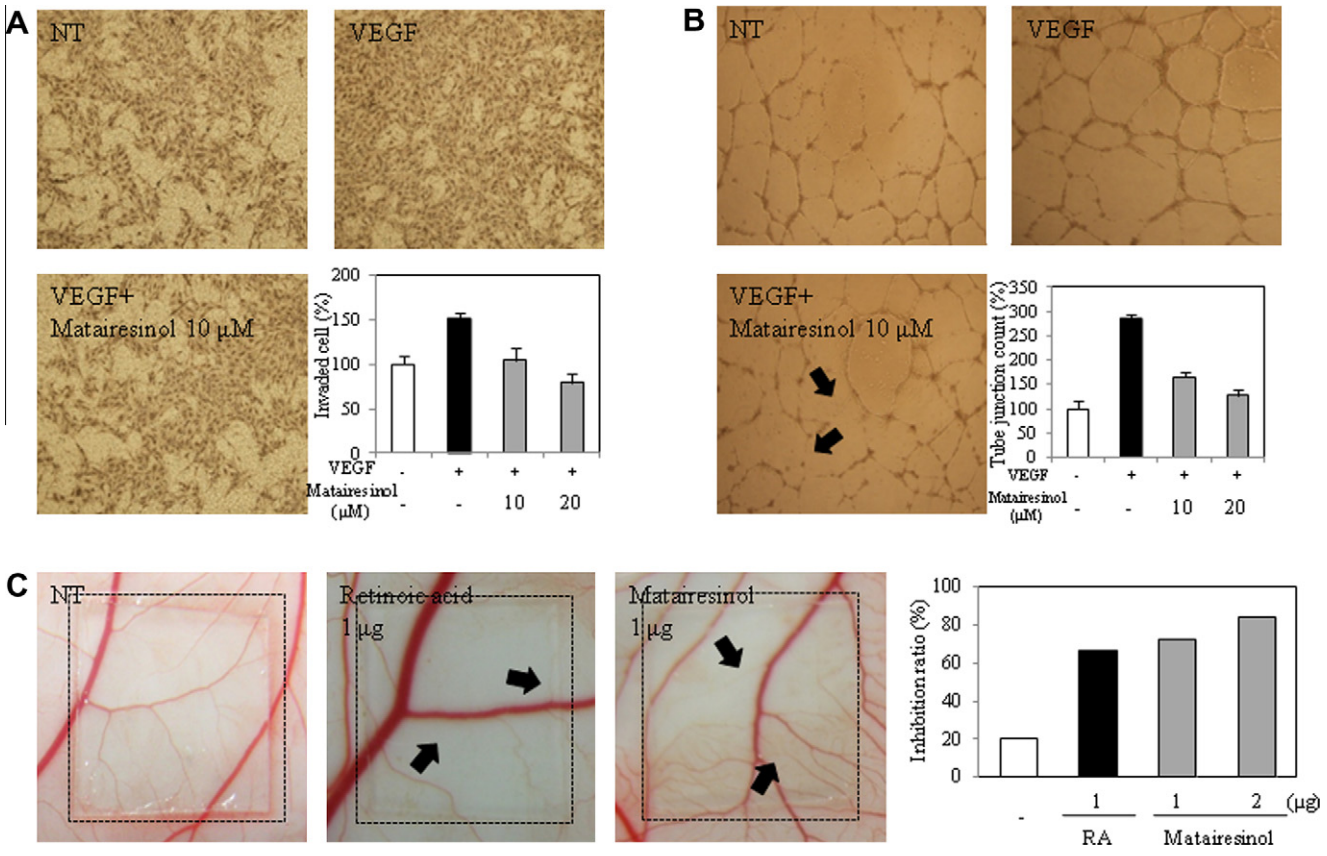

Fig. 3. *In vitro* and *in vivo* anti-angiogenic activity of matairesinol. Serum-starved human umbilical vein endothelial cells (HUVECs) were stimulated with vascular endothelial growth factor (VEGF) (30 ng/mL) in the presence or absence of matairesinol. (A) Inhibitory activity of matairesinol on endothelial cell invasion. (B) Effect of matairesinol on the tube-forming ability of HUVECs. Arrows indicate broken tubes formed by VEGF-stimulated HUVECs. The basal level of invasion (A) and capillary tube formation (B) of HUVECs in serum-free media were normalized to 100%. (C) Anti-angiogenic activity of matairesinol *in vivo* (a) EtOH control, (b) RA (1 μg/egg), (c) matairesinol (1 μg/egg), and (d) matairesinol (2 μg/egg) were applied to the chorioallantoic membrane (CAM), and the membrane was observed. Arrows indicate matairesinol-mediated neovascularization inhibition of CAM. Calculations were based on the proportion of positive eggs relative to the total number of eggs tested.

control and test groups. A *p*-value less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Crude extract of Cedrus deodara G. Don and matairesinol suppress mROS generation

We found that *Cedrus deodara* G. Don crude extract dose-dependently suppressed hypoxia-induced mROS generation in HeLa cells (Fig. 1B). Matairesinol is an active principal component of this extract (Fig. 1A). Therefore, we investigated whether matairesinol was responsible for the extract's suppression of mROS generation. As shown in Fig. 1C, matairesinol inhibited mROS generation during hypoxia in a dose-dependent manner. These experiments identify matairesinol as a new, natural small molecule that suppresses mROS.

3.2. Matairesinol potently inhibits HUVEC proliferation

We next investigated the effect of matairesinol on HUVEC and HeLa cell proliferation. These cells were treated with various concentrations of matairesinol for 3 days, and cell growth was assessed by the MTT colorimetric assay. Notably, matairesinol inhibited cell growth more strongly in HUVECs (IC_{50}, 75 μM) than HeLa cells (IC_{50}, 200 μM) (Fig. 2A). To determine the optimum dose of matairesinol that did not cause cytotoxic side effects, various concentrations of matairesinol (1–100 μM) were applied to HUVECs, and cell viability was determined using the trypan blue exclusion method. Matairesinol did not cause HUVEC cytotoxicity at doses up to 50 μM for 3 days. Therefore, the following studies were performed using a concentration range of 5–20 μM (Fig. 2B).

3.3. Matairesinol inhibits in vitro and in vivo angiogenesis

Next, we investigated the effect of matairesinol on HUVEC angiogenic phenotypes, including chemoinvasion and tube formation. Serum-starved HUVECs were stimulated by VEGF with or without matairesinol. As shown in Fig. 3A, matairesinol inhibited VEGF-induced HUVEC invasiveness in a dose-dependent manner without cytotoxic effects. The effect of matairesinol on HUVEC tube formation activity was also investigated. Matairesinol inhibited VEGF-induced HUVEC tube formation in a dose-dependent manner (Fig. 3B). These data indicate that matairesinol effectively inhibits VEGF-induced angiogenesis *in vitro*.

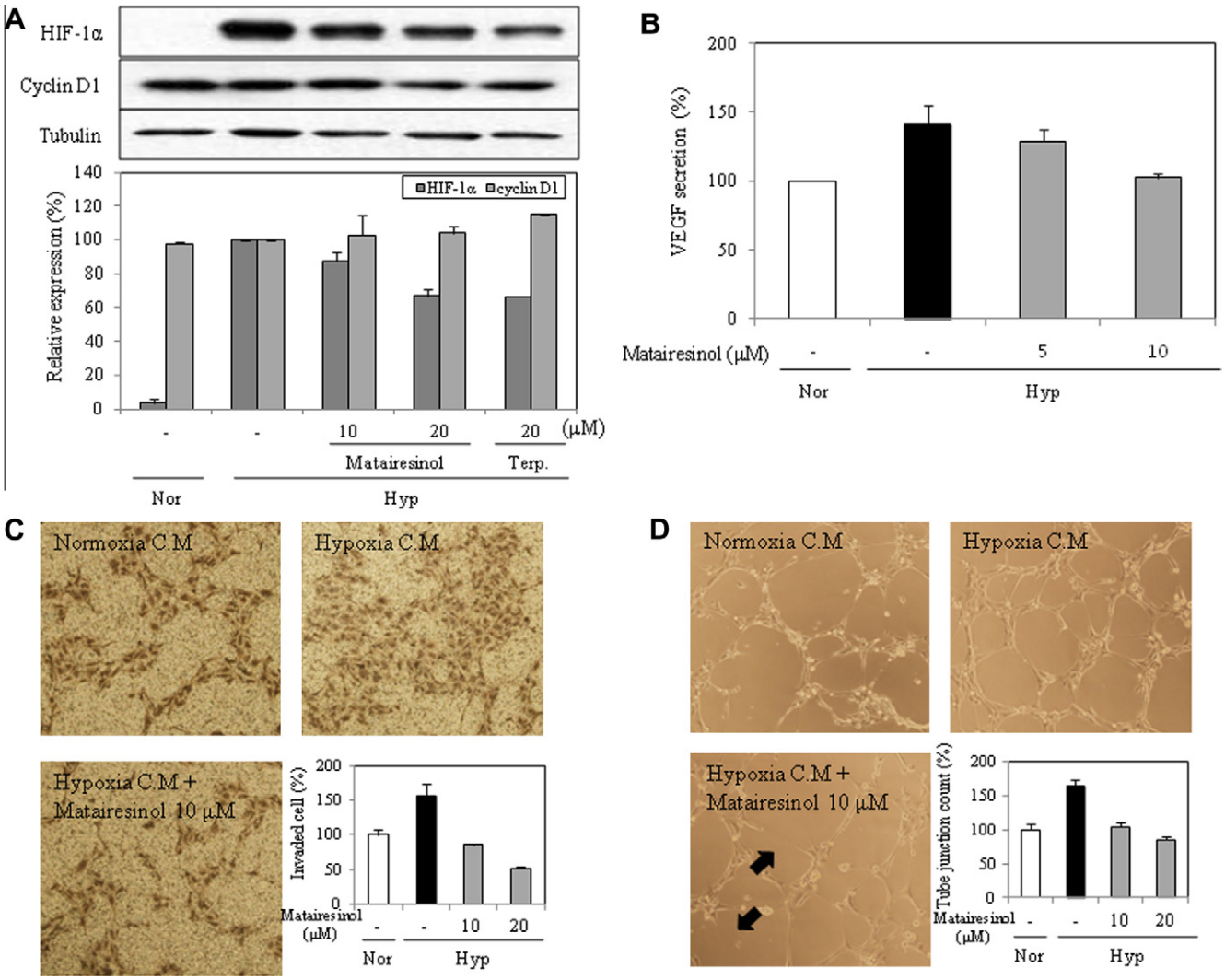

Fig. 4. Effect of matairesinol on angiogenic factor expression. (A) Hypoxia-inducible factor-1α (HIF-1α) and cyclin D1 expression levels were detected by Western blot. The level of tubulin was used as an internal control. (B) The expression level of vascular endothelial growth factor (VEGF) protein in HeLa cells was determined with an immunoassay. (C, D) Tumor conditioned media-induced angiogenesis of invasion (C) and tube formation (D). HUVECs were seeded in the upper chamber, and HeLa cell-conditioned medium was added to the lower chamber without VEGF. Nor, Normoxia; Hyp, Hypoxia.

Furthermore, the antiangiogenic activity of matairesinol was evaluated *in vivo* by using the chick embryo CAM assay. While normally developed CAMs exhibit extensive networks of capillaries, matairesinol-treated CAMs showed capillary formation without any sign of thrombosis or hemorrhage (Fig. 3C). These results demonstrate that matairesinol potently inhibits angiogenesis both *in vitro* and *in vivo* without cytotoxic effects.

3.4. Matairesinol suppresses HIF-1α stabilization and its target gene, VEGF, and inhibits tumor conditioned media-induced angiogenesis in vitro

HIF-1α plays a key role in tumor angiogenesis by regulating the expression of angiogenic factors, including VEGF, platelet-derived growth factor BB, and angiopoietin 2 [15,16]. Moreover, increased mROS generation triggers HIF-1α stabilization during hypoxia [17]. Therefore, we next examined the effect of matairesinol on HIF-1α expression levels under hypoxic conditions. Hypoxia-induced accumulation of HIF-1α protein was dose-dependently reduced by matairesinol without affecting the synthesis of cytoskeletal (tubulin) or cell cycle (cyclin D1) proteins (Fig. 4A). Terpestacin, which is known to block the generation of mROS and attenuate HIF-1α expression, was used as a positive control [10]. Downstream of reduced HIF-1α expression, matairesinol treatment inhibited the hypoxia-induced expression of VEGF, a HIF-1α target gene, in a dose-dependent manner (Fig. 4B). Moreover, tumor conditioned media-induced invasiveness (Fig. 4C) and HUVEC tube formation (Fig. 4D) was inhibited by matairesinol in dose-dependent manner. These results demonstrate that matairesinol potently inhibits tumor conditioned media-induced angiogenesis through suppression of the angiogenic factor, HIF-1α, without any cytotoxic effects.

The results of this study clearly demonstrate that matairesinol, an active principal component of *Cedrus deodara* (Roxb) G. Don extract, suppresses hypoxia-induced mROS generation and exhibits anti-angiogenic activity both *in vitro* and *in vivo*. In HUVECs, the expression levels of HIF-1α and its target gene, VEGF, were dose-dependently suppressed by matairesinol. In addition, matairesinol inhibited tumor conditioned media-induced invasiveness and HUVEC tube formation in a dose-dependent manner. Collectively, these results suggest that this compound might provide the basis for the development of anti-angiogenic agents with a unique mode of action. It is noteworthy that matairesinol effectively suppresses hypoxia and VEGF-induced angiogenesis at lower doses than are necessary to inhibit HUVEC growth, suggesting that the compound may specifically perturb angiogenic signaling pathways via the suppression of mROS generation. Further investigations on identifying and validating compound targets and its effect on mitochondria will elucidate the interesting biological activities of the compound. In conclusion, matairesinol is a new angiogenesis inhibitor that might provide a basis for developing novel anti-angiogenic therapeutics that target mROS-mediated signaling.

Acknowledgments

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