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Original article

Toluhydroquinone, the secondary metabolite of marine algae symbiotic microorganism, inhibits angiogenesis in HUVECs

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ABSTRACT

Angiogenesis, the growth of new blood vessels from the existing ones, occurs during embryo development and wound healing. However, most malignant tumors require angiogenesis for their growth and metastasis as well. Therefore, inhibition of angiogenesis has been focused as a new strategy of cancer therapies. To treat cancer, there are marine microorganism-derived secondary metabolites developed as chemotherapeutic agents. In this study, we used toluhydroquinone (2-methyl-1,4-hydroquinone), one of the secondary metabolites isolated from marine algae symbiotic fungus, *Aspergillus* sp. We examined the effects of toluhydroquinone on angiogenesis using HUVECs. We identified that toluhydroquinone inhibited the activity of β-catenin and down-regulated Ras/Raf/MEK/ERK signaling which are crucial components during angiogenesis. In addition, the expression and activity of MMPs are reduced by the treatment of toluhydroquinone. In conclusion, we confirmed that toluhydroquinone has inhibitory effects on angiogenic behaviors of human endothelial cells, HUVECs. Our findings suggest that toluhydroquinone can be proposed as a potent anti-angiogenesis drug candidate to treat cancers.

1. Introduction

As novel bioactive secondary metabolites isolated from marine microorganisms have anti-tumor, anti-virus and other bioactive properties, investigation of secondary metabolites from marine microorganisms has been an attractive field to search potent drug candidates [1,2]. This is the first report that investigates anti-angiogenesis effects of toluhydroquinone, derived from marine algae symbiotic fungus, *Aspergillus* sp. (Fig. 1) [3].

Angiogenesis is strictly regulated via multi-step processes, involving endothelial cell proliferation, invasion, migration and tube formation [4]. Especially, tumor neovascularization, expansion of a network of blood vessels penetrating into tumor, is essential for tumor growth and metastasis throughout the body [4,5]. Therefore, inhibition of angiogenesis has been focused as novel anti-cancer strategy to regulate tumor growth and metastasis [6].

Ras activation gives an angiogenic phenotype to endothelial cells [7]. Ras can control endothelial cell proliferation and migration via Raf/MAPK pathway stimulated by pro-angiogenic factors such as VEGF, bFGF or PDGF [8–11]. In this pathway, Ras particularly phosphorylates Raf, MEK1 and MEK-2, which then phosphorylate Thr and Tyr residues within the activation loop of kinase domain of ERK-1 and ERK-2 [12].

Endothelial cells secret matrix metalloproteases (MMPs) which belong to family of zinc-dependent proteases that can proteolytically digest specific extracellular matrix components. Endothelial cells invade into the surrounding matrix, creating space for a vascular lumen. Therefore, proteolysis of matrix components is

Abbreviations: HUVECs, human umbilical vein endothelial cells; MEK, MAPK kinase; ERK, extracellular signal-regulated kinases; MMPs, matrix metalloproteinases; VEGF, vascular endothelial cell growth factor; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; VE-cadherin, vascular endothelial-cadherin; AJs, adherens junctions; ECM, extracellular matrix; MAPK, mitogen-activated protein kinases; PKA, cAMP-dependent protein kinase; PKB, protein kinase B.

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required to initiate angiogenesis [13,14]. Several MMPs including MMP-2 and MMP-9 have been reported as crucial proteases for angiogenic switch [15]. The activities of MMP-2 and MMP-9 facilitate endothelial cell invasion, leading endothelial cell survival and/or migration and influence on release of pro-angiogenic factors or destruction of angiogenesis inhibitors [16].

β-catenin functions in not only intercellular adhesion, called adherens junctions (AJs), but also Wnt signaling in the endothelial cells [17]. β-catenin and VE-cadherin form AJ complex, which is essential for morphogenesis in endothelial cells [18,19]. In addition, β-catenin is an effector protein of Wnt signaling in endothelial cells. In the presence of Wnt signaling, β-catenin forms a complex with transcription factors such as T-cell factor (TCF) and Lymphoid enhancer-binding factor (LEF) to trigger activation of target genes [20,21]. However, under the absence of Wnt signaling, β-catenin is constitutively phosphorylated by Glycogen-synthase kinase 3β (GSK-3β) at several serine and threonine residues leading to degradation of β-catenin by proteasome [22,23].

In this study, we observed that toluhydroquinone inhibited cell adhesion, invasion, migration and tube-like structure formation through regulation of various factors including MMPs, β-catenin and Ras/MAPK signaling. Therefore, we expect that these novel effects on anti-angiogenic mechanisms suggest its potential therapeutic application in cancer metastasis..

2. Materials and methods

2.1. Fungal isolation and culture

The fungal strain, *Aspergillus* sp. was isolated from the surface of the marine red alga *Hypnea saidana* collected in Tongnyeong, Gyeongnam province, Korea, and identified based on the morphological evaluation. A voucher specimen is deposited at Pukyong National University with the code MFA292. The fungus was cultured (20 L) for 21 days (static) at 29 °C in SWS medium: soytone (0.1%), soluble starch (1.0%), and seawater (100%).

2.2. Extraction and isolation

The culture broth and mycelium were separated, and the broth (10 L) was extracted with ethyl acetate to provide a crude extract (640 mg) which was subjected to silica gel flash chromatography and progressively eluted with n-hexnae/ethyl acetate (5:1), n-hexane/ethyl acetate (1:1), n-hexane/ethyl acetate (1:5), n-hexane/ethyl acetate (1:10) and finally with ethyl acetate. Each collection (30 mL each) was combined on the basis of their thin-layer chromatography (TLC) profiles to yield five major fractions. Medium pressure liquid chromatography (MPLC) of each fractions on ODS column by elution with methanol afforded five crude compounds, respectively. The isolated crude compounds were further purified by high-performance liquid chromatography (HPLC) (YMC ODS-A, methanol) utilizing a 30 min gradient program of 50% to 100% methanol in H_2O to furnish (+)-epoxydon (5.0 mg), (+)-epoxydon monoacetate (12.0 mg), gentisyl alcohol (10.0 mg), 3-chlorogentisyl alcohol (20.0 mg), and toluhydroquinone (5.0 mg), respectively. The isolated toluhydroquinone was used in this study.

2.3. Physicochemical data of toluhydroquinone

Toluhydroquinone: $C_7H_8O_2$ (Fig. 1). A colorless needle; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.44 (1H, s, 2-OH), 6.53 (1H, d, J = 8.5 Hz, H-3), 6.36 (1H, dd, J = 8.5, 2.8 Hz, H-4), 8.48 (1H, s, 5-OH), 6.45 (1H, d, J = 2.8 Hz, H-6), 2.02 (3H, s, H_3-7); ^{13}C NMR (100 MHz, DMSO-d_6) δ 124.3 (s, C-1), 147.6 (s, C-2), 117.1 (d, C-3), 115.0 (d, C-4), 149.5 (s, C-5), 112.6 (d, C-6), 16.1 (q, C-7).

2.4. Experimental reagents

Toluhydroquinone stock solution (50 mM) was prepared by dissolving in dimethyl sulfoxide (DMSO) and stored at −20 °C. The stock solution was further diluted with the appropriate assay medium immediately before use. The maximum final concentration of DMSO (< 0.1%) did not affect cell proliferation and did not induce cytotoxicity on the cell lines (data not shown). VEGF, methanol and DMSO were purchased from Sigma-aldrich (Saint-Louis, MO, USA), formaldehyde (Junsei, Tokyo, Japan), Giemsa (Gurr-Giemsa, BDH Merk Ltd, Poole, England) and WST-1® (Daeil Lab service, Jong-No, Korea) were used. EGM-2 medium was obtained from Lonza (Walkersville, MD, USA) and fetal bovine serum (FBS) was obtained from Cellgro (Manassas, VA, USA). All rabbit monoclonal antibodies against MMP-9, MMP-2, Ras, c-Raf, p-c-Raf (S259), p-MEK1/2 (S217/221), ERK 2, p-ERK 1/2 (Y202/T204), Rac1, β-catenin, p-β-catenin (S675 and S552), p-GSK-3β (S9), p-Akt (S473) and β-actin and mouse monoclonal antibody GSK-3β were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA).

2.5. Cell culture

Cell culture were prepared and maintained according to standard cell culture procedures. Human vascular endothelial cells (HUVECs) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in an endothelial basal medium-2 (EBM-2) supplemented with EGM-2 singleQuots kit (Lonza Group Ltd, walkersville, MD, USA), 1% (*v*/*v*) penicillin-streptomycin (PAA Laboratories GmbH, Austria) and 10% (*v*/*v*) fetal bovine serum (FBS) at 37 °C and 5% CO_2. HUVECs between P2 and P5 were used for all experiment.

2.6. Tube-like structure formation assay

The tube formation assay was used to investigate the effect of toluhydroquinone on angiogenesis in vitro. Each well of Lab-Tek chamber slides (Thermo Fisher Scientific, Rochester, NY, USA) was coated with 200 μl liquid Matrigel (Corning Incorporated Life Sciences, Tewksbury, MA, USA), which was allowed to solidify at 37 °C for 30 min. HUVECs were seeded at a density of 5×10^4 cells in culture medium containing different concentration of toluhydroquinone (0∼15 μM). The cells were incubated for 12 h at 37 °C and 5% CO_2 (sufficient for formation of an intact network in the control group), and the tube-like structure formation was observed, using the phase contrast inverted microscope (Olympus CKX41; Olympus Optical Co. Ltd, Tokyo, Japan). Total vessel lengths were counted with Wimasis imaging analysis software (Wimasis GmbH, Munich, Germany).

2.7. Cell proliferation assay

HUVECs (1×10^4 cells/well) were seeded on 96-well culture plates and incubated for 24 h, then the various concentrations of toluhydroquinone (0∼90 μM) were added to the culture medium. After 24 h, 10 μl of WST-1 solution was added to each well, and the cells were incubated at 37 °C in an atmosphere of 5% CO_2 for 3 h. The absorbance was measured at 460 nm with the microplate reader (Molecular Devices, Silicon valley, CA, USA). Experiments were performed in triplicate.

2.8. Adhesion assay

Plates were coated with 5 μg/cm^2 Collagen, Fibronectin (BD Biosciences, Two Oak Park, Bedford, MA USA) or 50 μg/mL Matrigel on the bottom of 96-well plates and overnight at 4 °C. Plates were