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Curcumin inhibits the formation of capillary-like tubes by rat lymphatic endothelial cells

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Abstract

The natural pigments curcumin and berberine have been shown to exhibit a variety of pharmacologic effects including anti-inflammatory, anti-cancer, and anti-metastatic properties. Here, we investigated the anti-lymphangiogenic effect with an *in vitro* tube-forming model using conditionally immortalized lymphatic endothelial TR-LE cells, a newly established cell line originating from the thoracic duct of a transgenic rat expressing the temperature-sensitive SV40 large T-antigen. Curcumin, but not berberine, exhibited a dose-dependent inhibition of the formation of capillary-like tubes by TR-LE cells without affecting cell viability and adhesion to Matrigel. To address the molecular mechanisms involved, we performed experiments with specific inhibitors against putative targets of curcumin, including IκB kinase (IKK), epidermal growth factor receptor (EGFR), phosphatidylinositol-3 kinase (PI3K)/Akt, and matix metalloproteinases (MMPs). While the IKK-2 inhibitor VI and EGFR tyrosine kinase inhibitors gefitinib and PD153035 had no effect, both the PI3K inhibitor LY294002 and the MMP inhibitor GM6001 shortened the tubes by approximately 50%. Western blot analysis and gelatin zymography revealed that curcumin, but not berberine, has an inhibitory effect on the phosphorylation of Akt and enzymatic activity of MMP-2 in TR-LE cells. These results suggest that curcumin exerts its inhibitory effect on lymphangiogenesis partly through Akt and MMP-2.

Keywords: Curcumin; Berberine; Akt; MMP-2; Lymphatic metastasis; Lymphangiogenesis

1. Introduction

Curcumin is a naturally occurring yellow pigment from the rhizome of the perennial herb *Curcuma longa*. Turmeric, the powdered form of the rhizome, has been used for centuries in traditional medicine. Moreover, curcumin has been reported to block nuclear factor κB (NF-κB), IκB

Abbreviations: EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; MMP, matix metalloproteinase; PI3K, phosphatidylinositol-3 kinase; SV40, simian virus 40; TNF, tumor necrosis factor; VEGFR, vascular endothelial growth factor receptor.

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kinase (IKK), epidermal growth factor receptor (EGFR), the Akt pathway, and matrix metalloproteinases (MMPs) expression, and shown to exhibit a variety of pharmacologic effects including anti-inflammatory, anti-infectious, anti-angiogenic, and anti-cancer activities [1–3]. Berberine, an isoquinoline alkaloid and an active component of *Berberis aquifolium* (Oregon grape), *Berberis aristata* (tree tumeric), *Berberis vulgaris* (barberry), *Coptis chinensis* (coptis or goldenthread), and *Hydrastis canadensis* (goldenseal), also has a wide range of pharmacological and biochemical effects including anti-microbial, anti-diarrhea, anti-inflammatory, anti-angiogenic, and anti-cancer activities [4–6]. In our previous studies, both curcumin and berberine markedly inhibited the mediastinal lymph node metastasis produced by the orthotopic implantation of Lewis lung carcinoma (LLC) cells, their effects correlating with the inhibition of cell invasion and modulation of transcription factor activator protein-1 (AP-1) in LLC cells [7,8]. However, the mechanisms of this anti-metastatic activity are not fully understood.

In the past, it was thought that lymphatic metastasis was a passive process in which detached tumor cells reached lymph nodes via drainage through pre-existing local lymphatic vessels [9]. However, recent studies have identified lymphangiogenic growth factors and histochemical markers that discriminate between blood vessels and lymphatics, revealing that lymphangiogenesis plays a critical role in metastasis [10,11]. The expression of lymphangiogenic growth factors in a range of animal tumor models leads to the formation of lymphatic vessels either within or at the periphery of the tumors and this is accompanied by enhanced lymphatic metastasis and, in some cases, by metastasis to distant organs [11]. Thus, a culture of lymphatic endothelial cells would help us to elucidate the mechanisms of lymphangiogenesis *in vitro*. Recently, we succeeded in establishing a rat lymphaticendothelial cell line (TR-LE) from the thoracic duct of a transgenic rat harboring a temperature-sensitive simian virus 40 (SV40) large T-antigen and enhanced green fluorescent protein (EGFP) [12]. TR-LE cells possess tube-forming ability on Matrigel and express the lymphatic endothelial markers VEGFR-3 (vascular endothelial growth factor receptor), LYVE-1 (a lymphatic endothelial receptor), Prox-1 (a homeobox gene product), and podoplanin (a glomerular podocyte membrane mucoprotein), together with the endothelial markers CD31, Tie-2, and VEGFR-2. This lymphatic endothelial cell line enables one to conduct experiments on lymphangiogenesis *in vitro*.

In this study, we assess the effect of curcumin and berberine on the formation of capillary-like tubes using TR-LE cells, and reveal potential targets of curcumin's the anti-metastatic activity.

2. Materials and methods

2.1. Materials

Curcumin and berberine were purchased from Wako Chemical (Osaka, Japan). GM6001, PD153035, and IKK-2 inhibitor VI were purchased from Calbiochem (Darmstadt, Germany). LY294002, TNF-α, and doxorubicin hydrochloride were purchased from Alomone Laboratories (Jerusalem, Israel), R&D systems, and Kyowa Hakko Induxtries, Ltd. (Tokyo, Japan), respectively. Gefitinib was kindly provided by AstraZeneca (Macclesfield, UK). Reagents were dissolved in dimethylsulfoxide or distilled water and stored at −20 °C.

2.2. Cells

TR-LE cells, a conditionally immortalized rat lymphatic endothelial cell line [12], were maintained on culture dishes, which had been pre-coated with 10 μg/mL fibronectin (Iwaki Glass, Tokyo, Japan), in HuMedia-EG2 (Kurabo, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Lot 9354F, ICN Biomedicals Inc., Aurora, OH) at a permissive temperature (33 °C). Lewis lung carcinoma (LLC) cells, kindly provided by Dr. K. Takeda (Juntendo University, Tokyo), were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS at 37 °C.

2.3. Tube formation assay

Sub-confluent TR-LE cells were harvested with trypsin–EDTA and centrifuged at 1000 rpm for 5 min. A cell suspension (2×10^4) was then prepared in DMEM supplemented with 10% FBS and seeded in a 96-well plate that had been pre-coated with 40 μl of 10 mg/mL Matrigel (Collaborative Research Co., Bedford, MA). After a 5-h incubation at 37 °C, cultures were fixed by using glutaraldehyde and stained with hematoxylin. The tube-like network was traced by using Biz-Tablet (WACOM, Saitama, Japan) and lengths were quantified with a ChemiDoc XRS system (Bio-Rad, Tokyo, Japan).

2.4. Cell proliferation assay

TR-LE and LLC cells (2×10^4 cells/well) were seeded in 100 μl of DMEM containing 10% FBS in 96-well plates. Cells were allowed to adhere for 2 h, and then 25 μl of medium containing curcumin, berberine, and