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Cucurbitacin B inhibits breast cancer metastasis and angiogenesis through VEGF-mediated suppression of FAK/MMP-9 signaling axis

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ABSTRACT

Available breast cancer therapeutic strategies largely target the primary tumor but are ineffective against tumor metastasis and angiogenesis. In our current study, we determined the effect of Cucurbitacin B (CuB), a plant triterpenoid, on the metastatic and angiogenic potential of breast cancer cells. CuB was found to inhibit cellular proliferation and induce apoptosis in breast cancer cells in a time- and dose-dependent manner. Further, CuB-treatment significantly inhibited the migratory and invasive potential of highly metastatic breast cancer MDA-MB-231 and 4T1 cells at sub-IC_{50} concentrations, where no significant apoptosis was observed. CuB was also found to inhibit migratory, invasive and tube-forming capacities of HUVECs *in vitro*. In addition, inhibition of pre-existing vasculature in chick embryo chorioallantoic membrane *ex vivo* further supports the anti-angiogenic effect of CuB. CuB-mediated anti-metastatic and anti-angiogenic effects were associated with the downregulation of VEGF/FAK/MMP-9 signaling, which has been validated by using FAK-inhibitor (FI-14). CuB-treatment resulted in a significant inhibition of VEGF-induced phosphorylation of FAK and MMP-9 expressions similar to the action of FI-14. CuB was also found to decrease the micro-vessel density as evidenced by the decreased expression of CD31, a marker for neovasculature. Further, CuB-treatment inhibited tumor growth, lung metastasis and angiogenesis in a highly metastatic 4T1-syngeneic mouse mammary cancer. Collectively, our findings suggest that CuB inhibited breast cancer metastasis and angiogenesis, at least in part, through the downregulation of VEGF/FAK/MMP-9 signaling.

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1. Introduction

Breast cancer is the leading cause of cancer-associated mortality among women. Treatment of breast cancer becomes difficult as the tumor cells metastasize into distant body organs. Initiation of angiogenesis plays a critical role in breast cancer progression, invasion, and metastasis. The neovascularisation offers cancer cells with its vital requirements while concurrently offering a way by which malignant cells can metastasize to the secondary site. Indeed, high tumoral vascularity has been demonstrated to be a prognostic factor in many human cancers including breast cancer (Brem et al., 1977; Jensen et al., 1982; Weidner, 1998). Transfection of cancer cells with pro-angiogenic peptides has been shown to augment tumor growth, invasiveness, and metastasis. Conversely, different inhibitors of angiogenesis have been shown to reduce tumor growth and metastasis (Ferrara and Kerbel, 2005). Hence, the therapeutic attention should be given to the combined treatment of primary tumor along with the progression of metastasis and angiogenesis.

Cucurbitacins are traditionally used medicinal compounds derived from plants of *Cucurbitaceae* family. Cucurbitacin B (CuB) is most abundantly available in plants such as *Trichosanthes cucumerina* L., *Cucurbita andreana etc.* (Alghasham, 2013). CuB has shown its tremendous anti-cancerous potential in many cancer subtypes

Abbreviations: bFGF, basic fibroblast growth factor; CuB, Cucurbitacin B; CAM, chorioallantoic membrane; ECM, extracellular matrix; FAK, focal adhesion kinase; HUVECs, human umbilical vein endothelial cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; VEGF, vascular endothelial growth factor.

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including breast cancer. CuB has been known to suppress breast cancer by inhibiting the expression of *hTERT* and *c-myc*, induction of G_2/M cell cycle arrest and apoptosis, disruption of F-actin filaments and, inhibition of HER2-integrin signaling (Duangmano et al., 2010, 2012a,b; Gupta and Srivastava, 2014; Thoennissen et al., 2009; Yar Saglam et al., 2016). We have recently reported the epigenetic modulatory activities of CuB in non-small cell lung cancer *in vitro* and *in vivo* (Shukla et al., 2015). However, the effect of CuB and their molecular mechanisms in regulation of breast cancer cell migration, invasion and angiogenesis have not been reported yet.

Several studies have revealed that elevated levels of focal adhesion kinase (FAK) expression contributes to the metastatic potential of breast tumor cells (Cance et al., 2000; Lark et al., 2005). Formation of neo-vasculature from the pre-existing blood vessels is the crucial event required for cancer progression and metastasis. Tumor angiogenesis is induced from the tumor cells through the production of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). These growth factors initiate a series of events by binding to their respective receptors thereby activating various signaling pathway. VEGF exerts its biological effects by binding to either of the two cell surface receptors, VEGFR1 or VEGFR2 leading to the activation of various cytoplasmic proteins including FAK and secretion of matrix metalloproteinases (MMPs) by tumor-associated endothelial cells, resulting in the development of neo-vasculature. The degradation of extracellular matrix (ECM) by MMPs mainly MMP-2 and MMP-9 have been consistently correlated with migration, invasion as well as angiogenesis in many cancer subtypes including breast cancer (Shibata et al., 1998). FAK activation has been reported to induce cell invasion by increasing MMP-2 and MMP-9 in tumor cells (Hsia et al., 2003; Schlaepfer et al., 2004). The FAK activity has been shown to be directly associated with the VEGF-mediated vasculature in 4T1 mammary tumors compared to controls which signifies its role in angiogenesis (Peng et al., 2004; Shen et al., 2005; Haskell et al., 2003).

Therefore in the present study, we investigated the anti-metastatic and anti-angiogenic effects of CuB on the highly metastatic human breast cancer MDA-MB-231 and mouse mammary cancer 4T1 cells. Effect of CuB on angiogenesis was studied using human umbilical vein endothelial cells (HUVECs) as *in vitro* model, and in chorioallantoic membrane (CAM) and matrigel plug assays as *ex vivo* and *in vivo* models, respectively. Further, we assessed the antitumor and antimetastatic effects of CuB using 4T1-syngeneic mouse mammary cancer model. We found that CuB blocked the VEGF-mediated tyrosine phosphorylation of FAK, which otherwise results into an adequate assembly of focal adhesions, and activation of MMPs leading to increased metastasis and angiogenesis *in vitro* and *in vivo*. These results provide an insight into the mechanism involved in the CuB-mediated suppression of breast cancer metastasis and angiogenesis.

2. Materials and methods

2.1. Cell culture and treatments

All the breast cancer cells were procured from ATCC, Manassas, VA. HUVECs were purchased from life technologies, Carlsbad, CA. Breast cancer cells were cultured in RPMI-medium supplemented with 10% FBS and 1% penicillin/streptomycin solution (Sigma-Aldrich, St. Louis, MO). HUVECs were cultured in 200 PRF-medium supplemented with 2% low serum growth supplements (Life Technologies), 10% FBS and 1% penicillin/streptomycin solution. CuB was isolated from the fruits of *L. graveolense* Roxb. and stored at a stock concentration of 10 mM, as described previously (Shukla et al., 2015). The structure and HPLC data of isolated CuB is given in Supplementary Fig. S1. FI-14 (Tocris Bioscience, Ellisville, MO) was dissolved in DMSO at a stock concentration of 10 mM and stored at −20 °C. Recombinant human and mouse VEGF proteins (Sigma-Aldrich) were reconstituted according to the manufacturer's instructions.

2.2. Cellular viability assay

Approximately 5×10^3 breast cancer MDA-MB-231, 4T1 cells and HUVECs were seeded in 96-well plates and treated with different concentrations of CuB for 24, 48 and 72 h. At the end of the incubation time, MTT solution (0.5 mg/mL) was added to each well and incubated for 2 h. The media containing MTT solution was aspirated from the wells and the MTT-formazan crystals were dissolved in DMSO. Absorbance was recorded at 540 nm wavelength. Results were represented as percent proliferation over control.

2.3. Annexin V-Alexa fluor and PI staining for cellular apoptosis analysis

MDA-MB-231 and 4T1 cells were plated in 6-well plates at a density of approximately 2×10^5 cells per well. Cells were then treated with indicated concentrations of CuB for 24 h. The samples were prepared using Alexa fluor® 488 annexin V/dead cell apoptosis kit (Invitrogen) according to manufacturer's instructions and were analyzed for apoptosis on FACSCalibur (BD Biosciences).

2.4. Wound healing assay

Wound healing assay was performed to assess the effect of CuB on migratory potential of MDA-MB-231, 4T1 and HUVEC cells as described previously (Khan et al., 2015). Briefly, 2×10^5 cells were plated in 6-well culture plates. A wound was created, and cells were treated with indicated concentrations of CuB. To examine the effect of CuB on VEGF-induced cell migration, cells were serum starved for 12 h, pretreated with VEGF (20 ng/mL) for at least 30 min, and then treated with CuB or FI-14 (1 μM) for another 24 h. The photographs were captured at 0, 12 and 24 h of post-treatment using Nikon's Eclipse TS100 microscope equipped with a digital camera. The wound area was measured using ImageJ software version 1.47 h (http://imagej.nih.gov/ij). Results were expressed as percent cell migration compared to respective controls.

2.5. Transwell invasion assay

To examine the effect of CuB on the invasive capacities of MDA-MB-231, 4T1 and HUVEC cells, transwell invasion assay was performed in Matrigel-coated invasion chambers following manufacturer's protocol (BD Biosciences), as described previously (Khan et al., 2015). Approximately, 2×10^4 cells were seeded on the upper well of Matrigel-coated chambers placed in 24-well plate in 450 μL of medium supplemented with 0.5% FBS containing indicated concentrations of CuB. To test the effect of CuB and FI-14 (1 μM) on VEGF-induced tube formation, 20 ng/mL of VEGF was added to each well. The lower chamber was filled with 750 μL medium supplemented with 10% FBS. Cells were incubated undisturbed in the CO2 incubator for 24 h. Thereafter, the invaded cells present on the lower surface of the membrane were fixed and stained with crystal violet (Sigma-Aldrich). The images were captured and the numbers of invaded cells were counted in at least ten different microscopic fields. Results were expressed as the percentage of invaded cells as compared to respective control.

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