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Chemically modified tetracyclines inhibit VEGF secretion by breast cancer cell lines

Mansi Kothari, Sanford R. Simon *

Department of Pathology, State University of New York at Stony Brook, Stony Brook, NY 11794-8691, USA

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Abstract

Chemically Modified Tetracyclines (CMTs) are antiproteolytic agents that have been shown to inhibit tumor invasiveness and metastasis. CMT 300 has shown promise in phase I clinical trials in patients with Kaposi's Sarcoma, which is characterized by over-production of the pro-angiogenic cytokine vascular endothelial growth factor (VEGF). In this study, we report a novel activity of CMT 308, a 9-amino derivative of CMT 300, on reducing levels of VEGF secreted by breast cancer cell lines. CMT 308, at sub-cytotoxic concentrations, reduced basal levels of secreted VEGF in the poorly invasive MCF-7 cell line as well as the more aggressively invasive MDA-MB-435s cell line in a dose-dependent manner. In addition, CMT 308 also reduced transforming growth factor β (TGFβ)-induced VEGF secretion in both cell lines. While VEGF could be detected in the conditioned media of untreated MCF-7 cells within 4 h, levels of secreted VEGF in CMT 308-treated cells remained undetectable up to 8 h. CMT 308 diminished secretion of VEGF from MCF-7 cells up to 8 h regardless of previous time in culture. CMT 308 did not reduce the levels of basal VEGF mRNA in either cell line, but did reduce pools of total intracellular VEGF protein. Although TGFβ stimulated an increase in VEGF levels in the conditioned media as well as in the cytoplasm, TGFβ treatment did not increase VEGF mRNA levels. Thus, augmented expression of VEGF protein by breast cancer cell lines in the presence of TGFβ appears to involve upregulation at a step beyond transcription. Moreover, the data strongly indicate that in these breast cancer cell lines, CMT 308 reduces VEGF secretion by targeting some post-transcriptional event. The capacity of CMT 308 to diminish levels of a major pro-angiogenic signal makes the nonantimicrobial tetracycline derivative an attractive candidate for anti-angiogenic therapy in management of breast cancer.
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1. Introduction

Breast cancer is the most frequently diagnosed non-skin cancer and ranks second among cancer deaths in women after lung cancer. Sustenance, progression and metastasis of breast and other tumors require elaboration of new micro-vessels from pre-existing vasculature through a process known as angiogenesis [1,2]. Angiogenesis is a consequence of a delicate balance between pro- and anti-angiogenic drives. Several pro-angiogenic molecules have been identified, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin 8 (IL-8), and transforming growth factor β (TGFβ) [3–5]. The most potent of these is VEGF, which is specifically mitogenic for endothelial cells that line blood vessel walls [6]. While molecules such as TGFβ are considered indirect inducers of angiogenesis, their role in promoting angiogenesis makes them an important target for anti-angiogenesis therapy [7,8]. One of the tumor-promoting functions of TGFβ is to induce angiogenesis by increasing VEGF production [9,10] in tumor cells. Moreover, there is evidence of the involvement of TGFβ in breast cancer. TGFβ levels are increased in the plasma of breast cancer patients, and these high TGFβ levels correlate with high

* Corresponding author. Fax: +1 631 444 7507.
E-mail address: ssimon@notes.cc.sunysb.edu (S.R. Simon).

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VEGF levels in the patients tested [11]. Hence, strategies that inhibit VEGF production by targeting regulatory molecules such as TGFβ have become viable candidates for anti-angiogenic therapy.

The focus of this study is a group of chemically modified tetracyclines (CMTs), non-antimicrobial tetracycline analogs that have anti-proteolytic properties [12]. The anti-proteolytic activity of CMTs may be responsible for their capacity to inhibit the invasiveness *in vitro* or *in vivo* of many neoplasms including glioblastoma; tumors of the breast [13], prostate [14–16], and colon [17,18]; and melanoma [19]. Additionally, the capacity of CMTs to induce apoptosis in a variety of cell lines has been proposed as a basis of their antitumor activity. Cells undergoing apoptosis in the presence of CMTs include macrophage tumor cell lines [20], leukemia cell lines [21], and prostate tumor cell lines [15]. Previous studies on CMTs have alluded to the antiangiogenic capacity of this subset of the tetracycline family of molecules. CMT 300 (6-deoxy-6-demethyl-4-dedimethylaminotetracycline), a modified tetracycline inhibits the formation of small tubules by human umbilical vein endothelial cells (HUVEC) [22]. CMT-1, which lacks the 4-dimethylamino group on the tetracycline ring system, appears to be more effective than minocycline in inhibiting endothelial cell proliferation [23]. Additionally, both, CMT 1 and CMT 8 (6α-deoxy-5-hydroxy-4-dedimethylaminotetracycline) inhibit the secretion of PMA-induced matrix metalloprotease 9 (MMP 9) expression in HUVEC [24]. Secretion of matrix degrading enzymes such as MMP-9 by HUVEC is thought to be important for formation of new microvessels during angiogenesis. Further evidence for the antiangiogenic effect of CMTs comes from the results of a phase I clinical trial with CMT 300 on patients with Kaposi's Sarcoma (KS). KS is generally considered a disease of overproduction of VEGF due to the presence of latent Human Herpes Virus 8 (HHV8) genes [25]. When treated with CMT 300, patients with KS had an overall response rate of 44 percent [26] and their lesions had visibly regressed [27]. Current anti-angiogenic therapies such as the recently approved drug Avastin involve neutralizing antibodies against VEGF protein. In this study, we report a novel capacity of CMT 308, a non-phototoxic, 9-amino derivative of CMT 300, to reduce secreted levels of the pro-angiogenic cytokine, VEGF, through a post-transcriptional modulatory mechanism, leading to diminished release of VEGF by breast cancer cells.

2. Materials and methods

2.1. Reagents

All reagents unless otherwise specified were purchased from Sigma (St. Louis, MO). MCF-7 cells, MDA-MB-435s cells, and Eagle's minimum essential medium (MEM) were purchased from ATCC (Manassas, VA). Dulbecco's minimum essential medium (DMEM)—low glucose and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT). TGFβ1 isolated from human platelets was purchased from Calbiochem (LaJolla, CA).

2.2. Cell culture

MCF-7 cells were maintained in Eagle's minimum essential medium (ATCC, Manassas, VA) supplemented with 0.01 mg/ml bovine insulin (Sigma–Aldrich, St. Louis, MO) and 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Sigma–Aldrich). MDA-MB-435s cells were maintained in DMEM-low glucose supplemented with 10% FBS and 1% penicillin/streptomycin.

2.3. Cytotoxicity assays

Fresh serum-free medium was added to cells followed by addition of MTS solution (Promega, Madison, WI). Cells were then incubated at 37 °C for 1–4 h, and absorbance was recorded at 490 nm.

2.4. VEGF quantitation in conditioned medium

MCF-7 and MDA-MB-435s cells (ATCC) were plated at 10^5 cells/well in 1 ml complete medium in 48-well plates and incubated overnight. The next day, medium was aspirated and replaced with fresh serum-free medium. Where indicated, TGFβ1 diluted in serum-free medium was added at various indicated concentrations. CMTs were diluted in DMSO and added to wells so that the final concentration of DMSO was 0.5%. Cells were further incubated for 24 h. Conditioned medium was then collected and stored at −20 °C. The VEGF Quantikine ELISA assay (R & D Systems, Minneapolis, MN) was used to assay VEGF in the conditioned medium from each sample. Loss of viability of the cells after the various treatments was evaluated by addition of MTS to a set of identically treated wells in the same plate.

2.5. VEGF quantitation in cell lysates

10^6 MDA-MB-435s cells plated in 10 ml serum-free medium were treated for 24 h with agents being tested or with DMSO as a vehicle control. Conditioned medium from the cells was collected for VEGF analysis. Cells were then trypsinized and lysed using 100 μL lysis buffer (150 mM sodium chloride, 40 mM Tris, 10% Glycerol, 0.1% NP40, 5 mM sodium fluoride, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, pH 7.8) with Complete Mini Protease Inhibitor Cocktail (Roche Biochemicals, Indianapolis, IN). Lysates were subjected to three freeze-thaw cycles centrifuged at 10,000 rpm for 10 min, and the supernatants collected for VEGF analysis. The VEGF Quantikine ELISA assay (R & D Systems, Minneapolis, MN) was used to assay VEGF in the lysates and conditioned medium from each sample. Twenty microliters aliquots of cell lysate or 150 μL aliquots of conditioned medium were routinely used for assay. Total

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