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Angiocidin inhibits breast cancer proliferation through activation of epidermal growth factor receptor and nuclear factor kappa (NF-κB)

Jessica Godek, Irene Sargiannidou [1], Sneha Patel, Lauren Hurd, Vicki L. Rothman, George P. Tuszynski *

Temple University School of Medicine, Center for Neurovirology, Department of Neuroscience, Philadelphia, PA 19140, USA

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ABSTRACT

Angiocidin, a tumor-associated peptide, has been previously shown to inhibit tumor progression by blocking angiogenesis. We now show that angiocidin has a direct inhibitory effect on tumor cell proliferation. MDA-MB-231 breast cancer cells were inhibited from proliferating in the presence of epidermal growth factor (EGF) and angiocidin. Angiocidin transfected breast cancer cells also displayed growth inhibition in vitro and failed to develop significant tumors in mice as compared to vector controls. The anti-proliferative effect of angiocidin was reversed by treating the cells with the epidermal growth factor receptor (EGFR) inhibitor 4557W, a potent tyrosine kinase inhibitor. Consistent with these results, we found that treatment of breast cancer cells with angiocidin induced a 2.3 fold increase in EGFR tyrosine 845 phosphorylation while no change in phosphorylation was observed in the remaining 16 phosphorylation sites of EGFR and those of its family members as measured by a human EGFR phosphorylation array. Treatment of breast cancer cells with angiocidin also resulted in the activation of nuclear factor B (Nf-κB) and the de novo up-regulation of many down-stream genes transcribed by Nf-κB, including cytokines, inflammatory mediators and the cell cycle inhibitor $p21^{waf1}$. Therefore, angiocidin is a peptide that not only inhibits tumor angiogenesis but also directly induces inhibition of tumor growth progression through the activation of EGFR and down-stream genes transcribed by Nf-κB.

Introduction

Angiocidin is a 41 kDa protein first isolated from lung carcinoma by affinity chromatography using the immobilized peptide CSVTCG present in the type 1 repeat region of thrombospondin-1, an important matrix protein that mediates tumor progression (Tuszynski et al., 1993). Angiocidin has a high degree of homology with two other proteins, anti-secretory factor (ASF) and S5a, with the exception of three additional amino acids at positions 769–771 in angiocidin (Zhou et al., 2004). ASF is a plasma-bound protein known to inhibit cholera toxin-induced intestinal fluid secretion in rats (Johansson et al., 1997). S5a is the polyubiquitin-binding subunit of the 26S proteasome, which is responsible for degradation of polyubiquitinated proteins (Walters et al., 2002). Like its homologue S5a, angiocidin also contains polyubiquitin-binding motifs, which have been shown to bind polyubiquitinated proteins on the surface of endothelial cells with high affinity (Dimitrov et al., 2005).

High levels of angiocidin have been found in the extracellular matrix of many types of cancers, including breast cancer, which suggests that it plays a role in tumor progression. Additionally, several studies have shown that recombinant angiocidin or its matrix binding domain has considerable anti-tumor activity (Sabherwal et al., 2006; Zhou et al., 2004). For example, in a mouse model of lung cancer iv injection of angiocidin reduced tumor growth by more than 90% (Zhou et al., 2004).

The precise mechanism of the anti-tumor activity of angiocidin has yet to be determined. Our previous studies suggest that angiocidin blocks tumor angiogenesis through its ability to bind extracellular matrix and block adhesion (Sabherwal et al., 2006) or block proteasome activity by competing with endogenous ubiquitin recognition subunits (Dimitrov et al., 2005). Recently, we made the unexpected discovery that when hematologic tumor cells such as leukemia cells were treated with angiocidin, they stop proliferating and undergo differentiation (Gaurnier-Hausser et al., 2008). This activity was due to activation of the Nf-κB pathway and the subsequent release of a cocktail of growth inhibitory and differentiation inducing cytokines that act on the cell in an autocrine manner

Abbreviations: Rel, C-Rel proto-oncogene protein; MAPK8IP3, C-jun-amino-terminal kinase-interacting protein 3; EGFR, Epidermal growth factor receptor; CSF2/GM-CSF, Granulocyte-macrophage colony-stimulating factor; IκBα, Inhibitor of Nf-κB; IFNG, Interferon gamma; IFNB1, Interferon beta 1; IL1B, Interleukin 1 beta; IL-8, Interleukin 8; MAP4K4, Mitogen-activated protein kinase kinase kinase kinase 4; MCP-1, Monocyte chemotactic protein-1 Nf-κB Nuclear factor kappa; Peli1, Protein pellino homolog 1; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NR2C2/TR4, Testicular receptor 4; TLR2, Toll-like receptor 2.

* Corresponding author. Temple University, Department of Neuroscience, 3500 North Broad Street, MERB-Room 754, Philadelphia, PA 19140, USA. Fax: +1 215 707 9977.
E-mail address: gpt@temple.edu (G.P. Tuszynski).
[1] Current address: University of Cyprus, The Cyprus Institute of Neurology and Genetics, P.O. Box 23462, 1683 Nicosia, Cyprus.

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(Gaurnier-Hausser and Tuszynski, 2009). Based on these data, we postulated that the same kind of mechanism might also explain in part the anti-tumor activity of angiocidin in solid tumors.

In this study we show that breast cancer cells treated with angiocidin undergo growth arrest and activation of EGFR and the Nf-κB pathway. We show that the consequence of this is inhibition of tumor growth in vivo. Remarkably, angiocidin just like in the leukemia system up-regulates Nf-κB responsive genes that inhibit proliferation directly through proteins such as the cell cycle inhibitor $p21^{waf1}$ or indirectly through secreted cytokines by an autocrine mechanism. These studies further define the anti-tumor activity of angiocidin.

Materials and methods

Materials

MDA-MB-231 cells were originally obtained from American Type Culture Collection (Manassas, VA). High glucose Dulbecco's modified Eagle Medium (DMEM) was obtained from Mediatech (Herndon, VA) and GIBCO, through Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS), bovine serum albumin (BSA), and L-glutamine were purchased from HyClone (Logan, UT). Rabbit monoclonal anti-p65, anti-IκBα, and anti-phospho-IκBα antibodies were obtained from Cell Signaling Technologies (Boston, MA). Rabbit monoclonal anti-phospho-p50 (Ser 337) was purchased from Santa Cruz (Santa Cruz, CA). Rabbit polyclonal anti-TLR2 antibody was purchased from Abcam (Cambridge, MA). Mouse monoclonal anti-p21 antibody was purchased from BioLegend (San Diego, CA). Mouse monoclonal anti-p53 antibody was obtained from CalBioChem (Gibbstown, NJ). Mouse monoclonal anti-β-actin antibody was obtained from Sigma-Aldrich (St. Louis, MO). Goat anti-rabbit IgG/horseradish peroxidase (HRP) and rabbit anti-mouse IgG/HRP conjugates were purchased from BioRad (Hercules, CA). Nuclear Extraction Kit was obtained from Panomics (Fremont, CA). Human CCL2/MCP-1 ELISA kit was obtained from eBioscience (San Diego, CA). RayBio Human Cytokine Antibody Array (Array 3) was purchased from RayBiotech, Inc. (Norcross, GA). RT² qPCR-Grade RNA Isolation Kit, RT² SYBR Green Master Mix, RT² First Strand Kit, and qRT-PCR array were purchased from SA Biosciences (Frederick, MD). For nuclear isolation and lysis, the Panomics Nuclear Extraction Kit was used (Affymetrix, Inc. Santa Clara, CA). Cells were lysed according to the manufacturer's protocol and stored at −80 °C.

Anchorage-independent growth

Soft agar assay was performed in 6 well plates where a base 0.8% Noble Agar (Difco Laboratories, MI) mixed with 10% FBS containing DMEM was coated. An aliquot of 5000 cells per well was mixed in 0.4% DMEM-agar and overlaid on the base agar. The plates were incubated at 37 °C in 5% CO_2 for 10–14 days. Colony formation was checked under the Olympus IMT-2 microscope with a 4× objective and digital images were obtained with a Kodak DC120 (Eastman Kodak, NY) camera device and the Photoenhanser software from PictureWorks Technologies.

Animal studies

Athymic mice were obtained from Charles River Laboratories and housed in the University's Animal Facility. The animals were nude/nude genotype and they lacked a functioning immune system so rejection of human cells did not occur. 6 female and aged matched animals per test group were injected on the right flank with 10^7 transfected cells as was previously reported (Zhou et al., 2004). Animals were examined every other day for tumor size as assessed with a caliper. The tumor volume was estimated using the formula $\text{length} \times \text{width}^2/2$. Six weeks later the animals were euthanized with CO_2 asphyxiation and the tumors were fixed, paraffin embedded, sectioned and examined by immunohistochemical staining for the expression of angiocidin in the tumors.

Cytokine and EGFR phosphorylation antibody array analysis

MB-231 cells were seeded in a 6-well plate and left until 75% confluent. Cells were then placed in 2% FBS DMEM for 2 h prior to a six-hour treatment or 24-hour treatment period with 10 μg/ml angiocidin for the cytokine array analysis and a 6 hour treatment with 10 μg/ml angiocidin for the EGFR array analysis. Untreated cells were maintained in 2% FBS without angiocidin for the same amount of time. Following treatment, media was collected from both treated and untreated samples and kept on ice until applying it to the antibody array membranes. Prior to application of media onto membranes, membranes were placed in 2 ml of blocking buffer for thirty minutes at room temperature. Media was then incubated with membrane overnight at 4 °C. Membranes were washed with provided wash buffers. Following the washes, membranes were incubated with biotin-conjugated anti-cytokine antibodies or biotin-conjugated anti-phospho EGFR antibodies in blocking buffer overnight at 4 °C. The next day, membranes were washed and incubated overnight at 4 °C with 2 ml of 1000-fold diluted HRP-conjugated streptavidin in blocking buffer. Following this overnight incubation, membranes were washed and detection buffers applied to the membranes. The membranes were then developed using autoradiography film.

PCR array analysis

In order to perform qRT-PCR of the Toll-like receptor (TLR) pathway genes, cells were either treated with 10 μg/ml angiocidin for 24 h or left untreated in 2% FBS DMEM. Cells were then lysed and RNA isolated using the protocol from the manufacturer (SABiosciences). RNA was isolated in a sterile, RNase-free environment. RNA concentration and purity were then determined using NanoDrop machinery and the quality of RNA was confirmed with an RNA gel. The RNA was then reverse-transcribed using the RT^2 First Strand Kit according to the manufacturer's protocol. One microgram of each sample was used for the first strand kit and was combined with a genomic DNA elimination mixture before undergoing reverse transcription polymerase chain reaction (PCR). The cDNA templates were then combined with SYBR green in the amount of 1.4 ml per sample of RNA. The cDNA/SYBR green mixture was applied in equal amounts (25 μl per well) to the 96-well plate, which contained primers for various TLR pathway genes. The plates were spun down at 600 rpm to rid the samples of bubbles that may have occurred due to pipetting. The plates were then kept on ice until running RT-PCR for 2.5 h. Following RT-PCR, data was analyzed using SABiosciences analysis program on the SABiosciences website.

Proliferation assay

Cell proliferation was measured using the Alamar blue assay as previously described (Zhou et al., 2004).

Sample preparation and protein assay

To perform cell lysis and protein collection, cells were washed twice with sterile phosphate-buffered saline (PBS). Cell Stripper was applied for a period of 20 min in 37 °C, 5% CO_2. Detached cells were spun at 700 rpm to obtain a cell pellet. Cells were lysed using either a 2% sodium dodecyl sulfate (SDS) lysis buffer or a 1% nonyl phenoxylpolyethoxylethanol-40 (NP-40) lysis buffer containing protease and phosphatase inhibitors and kept on ice for the duration of lysis. Lysates were then sonicated and spun at 15,000 rpm to obtain a