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Diallyl disulfide inhibits TNFα induced CCL2 release through MAPK/ERK and NF-Kappa-B signaling

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ABSTRACT

TNFα receptors are constitutively overexpressed in tumor cells, correlating to sustain elevated NFκB and monocyte chemotactic protein-1 (MCP-1/CCL2) expression. The elevation of CCL2 evokes aggressive forms of malignant tumors marked by tumor associated macrophage (TAM) recruitment, cell proliferation, invasion and angiogenesis. Previously, we have shown that the organo-sulfur compound diallyl disulfide (DADS) found in garlic (*Allium sativum*) attenuates TNFα induced CCL2 production in MDA-MB-231 cells. In the current study, we explored the signaling pathways responsible for DADS suppressive effect on TNFα mediated CCL2 release using PCR Arrays, RT–PCR and western blots. The data in this study show that TNFα initiates a rise in NFκB mRNA, which is not reversed by DADS. However, TNFα induced heightened expression of IKKε and phosphorylated ERK. The expression of these proteins corresponds to increased CCL2 release that can be attenuated by DADS. CCL2 induction by TNFα was also lessened by inhibitors of p38 (SB202190) and MEK (U0126) but not JNK (SP 600125), all of which were suppressed by DADS. In conclusion, the obtained results indicate that DADS down regulates TNFα invoked CCL2 production primarily through reduction of IKKε and phosphorylated-ERK, thereby impairing MAPK/ERK, and NFκB pathway signaling. Future research will be required to evaluate the effects of DADS on the function and expression of TNFα surface receptors.

1. Introduction

Metastatic breast cancer brings together rapid tumor proliferation, detachment and development of secondary tumors with acquired characteristics of the primary tumor. Chemokines such as monocyte chemotactic protein-1 (MCP-1), known as CC chemokine-2 (CCL2) play a critical role in this process. These chemokines recruit monocytes that differentiate into tumor-associated macrophages (TAMs) which subsequently release substances needed for tissue remodeling, angiogenesis and metastasis [1–3]. TAMs can also directly release more TNFα, an inflammatory cytokine [4], furthering the processes of production/release of CCL2 in diverse tumor tissue.[5].

Previously we have shown that diallyl disulfide (DADS), found in garlic (*Allium sativum*), attenuates TNFα induced CCL2 production in human breast cancer cells. It is likely that TNFα induced CCL2 production occurs through up regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). These three components are concurrently expressed to a greater degree in aggressively advanced tumors marked by TAM recruitment [6–8] and elevated TNF-receptors (TNFRs) on diverse human cancers [9]. Drugs or compounds such as DADS that antagonize these effects are becoming significant therapeutic vehicles. These include infliximab (Remicade), adalimumab (Humira), anti-TNF antibodies [10,11], all of which prevent tumor infiltrating leucocytes [12]. Therefore, the purpose of this study was to determine if the garlic constituent diallyl disulfide, could impact the MDA-MB-231 cells since they are the most studied TNBC to date. In addition, many researchers have used this cell model because it mimics aggressive nature of clinical isolates and have displayed great ability to metastasize in xenograft models. Nakagawa et al. [13] demonstrated the ability of DADS to inhibit tumor growth through its modulation of the apoptotic genes in this cell line. Other studies have used this cell line to study the mechanisms involved in the immune response [14,15]. Moreover, in this study we further explore the mechanism behind the inhibitory effect by DADS on TNFα induced CCL2 release in human breast carcinoma cells, with focus on MAPK/ERK NFκB signaling.

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2. Materials and methods

Cell lines, chemicals and reagents: Triple negative human breast tumor (MDA-MB-231) cells were obtained from American Type Culture Collection (Rockville, MD). Dulbecco's Modified Eagle Medium (DMEM) media, fetal bovine serum (FBS) and penicillin/streptomycin were all obtained from Invitrogen (Carlsbad, CA). Recombinant human TNFα was purchased from RayBiotech (RayBiotech Inc., Norcross, GA, USA). Diallyl disulfide (>80%) was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.1. Cell culture and treatment

MDA-MB-231 cells were cultured in 75 cm^2 or 175 cm^2 flasks containing DMEM media supplemented with 10% FBS and 1% pen/strep (10,000 U/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate). Cells were grown in an environment of 37 °C with humidified 95% air and 5% CO_2. Control cells received vehicle only (0.01% ethanol). DADS treated cells received 100 μM DADS in vehicle and 40 ng of TNFα was given to TNFα-treated and co-treated cells. Cells were incubated for 24 h. after treatment. In this study, 100 μM was used as the dose for DADS concentration. Although some studies have shown that different concentrations of garlic components exert different responses, such as increasing proliferation and tumor growth, in our laboratory cell viability studies were conducted to determine a working concentration and we recently reported that the 100 μM dose was the optimum dose for this cell line [16]. The present study is a continuation of the study performed by Bauer et al. [16]. Moreover, initial studies were done using both 100 μM and 400 μM with approximately the same effect on CCL2 release. Additionally in our previous studies, we demonstrated an optimum overall response using lower doses of DADS in MCF10A human epithelial cells [17]. Therefore, we used 100 μM since it would be less likely to have toxic effects on the normal cells at this dose level.

2.2. Inhibition study

Cultured MDA-MB-231 cells were treated for 24 h with DADS with and without TNFα treatment at above conditions. Additionally, cells were co-treated with inhibitors of JNK, MEK and p38 at concentrations of 10 μM, 2 μM and 2 μM, respectively. The inhibitors for JNK (SP600125), MEK (U0126) and p38 (SB202190) were purchased from Sigma Aldrich (St. Louis, MO). Cells were detached and the lysate collected.

2.3. ELISA: CCL2 detection

Supernatants from resting and stimulated (24 h) MDA-MB-231 cells were collected and centrifuged at 1000*g* for 5 min at 4 °C. Specific ELISA was performed using MCP-1/CCL2 ELISA kit (RayBiotech, Norcross, GA, USA) following manufacturer's instructions. Briefly, 100 μl of supernatants from samples and standards were added to 96 well plates pre-coated with capture antibody. After incubation 100 μl of prepared biotinylated antibody mixture was added to each well. After 1 h, mixture was decanted and 100 μl streptavidin solution was placed in each well and incubated. Substrate reagent (100 μl) was then added to each well for 30 min followed by the addition of 50 μl stop solution. Plates were read at 450 nm using a UV microplate reader.

2.4. Western blot: MAPK/ERK pathway and IKKE

Total cell protein concentrations from MDA-MB-231 cells treated with DADS, with and without TNFα co-treatment for 24 h, was determined using a modified Bio-Rad "DC" protein assay (Bio-Rad Laboratories, Hercules, CA, USA). A series of concentration standards ranging from 0 to 20 μg/ml were prepared using IgG. Test samples were prepared by adding 5 μl of a 1/10 dilution of the cell lysate to 795 μl H_2O. The standards and samples were mixed with 200 μl Bio-Rad "DC" protein assay dye concentrate and thoroughly mixed by vortexing. Following incubation for 5 min at room temperature, the samples were vortexed again and 200 μl of each loaded into a 96-well plate. Protein concentrations were quantified at a wavelength of 595 nm with the Power Wave X 340 microplate reader equipped with KC4 v3.0 PowerReports software (Bio-Tek Instruments, Winooski, VT, USA).

Cell lysates were separated by electrophoresis on 10% SDS–polyacrylamide gels and then transferred to Immobilon-P PVDF membranes. Equal loading was verified by staining with Ponceau S (Sigma–Aldrich Chemical Co., St. Louis, MO). Blots were blocked at 4 °C overnight in 5% Carnation Instant Milk in Tris-buffered saline with 0.05% Tween 20 in PBS (PBST) and then incubated overnight at 4 °C with mouse anti-human p38 MAPK, ERK and JNK affinity purified antibody (IMGENEX, San Diego, CA). Membranes were washed with PBST and incubated overnight with anti-goat IgG-horseradish peroxidase (Santa Cruz Biotechnology, CA) in PBST overnight at 4 °C. Protein loading was monitored in each gel lane by probing the membranes with anti-GAPDH antibodies (R&D Systems, Minneapolis, MN). Immunoblot images were obtained using a Flour-S Max Multimager (Bio-Rad Laboratories, Hercules, CA). Lane density data was acquired with Quantity One Software (Bio-Rad Laboratories, Hercules, CA).

2.5. RT–PCR

MDA-MB-231 cells treated with or without DADS, were subcultured in 6-well plates until confluent. Cells were lysed with 1 ml Trizol reagent. Chloroform (0.2 ml) was added to the lysed samples, tubes were shaken, incubated at 15–30 °C for 2–3 min and centrifuged at 10,000*g* for 15 min at 2–8 °C. The aqueous phase was transferred to a fresh tube and the RNA precipitated by mixing 0.5 ml isopropyl alcohol. After incubation the samples were centrifuged, the supernatant was removed and the RNA pellets were washed with 75% ethanol. The samples were mixed before being centrifuged at 7500*g* for 5 min at 2–8 °C. The RNA pellet was dried and dissolved in RNase-free water and incubated for 10 min at 55–60 °C.

RT reaction: RNA (5 μg/10 μl) was heated for 10 min then quenched on ice before use. The following components were added to the reaction: 10 μl heat denatured RNA, 3.0 μl 10× PCR buffer, 2.5 μl 10 mM dNTPs, 6.0 μl 25 mM $MgCl_2$, 1.0 μl random primers, 0.5 μl SuperScript II reverse transcriptase and 17.0 μl water. Samples were allowed to sit for 10 min at 25 °C then incubated for 1 h at 42 °C. The cDNA was denatured at 95 °C and placed on ice. PCR reaction: The following components were mixed in a 0.5 ml PCR tube: 6.0 μl cDNA product, 1.5 μl 10× PCR buffer, 0.2 μl Taq polymerase, 0.5 μl primer and 10.3 μl water. PCR will be performed with 30 cycles of denaturation: 30 s at 95 °C; annealing: 45 s at 60 °C; and extension 60 s at 72 °C using BioDoc-it System (UVP, Upland CA, USA). cDNA synthesis and Real-Time PCR was performed using First Strand cDNA synthesis kit/SABiosciences RT2 qPCR Master Mix from Qiagen (Gaithersburg, MD, USA) according to manufacturers instructions.

2.6. Statistical analysis

Statistical analysis on the data was determined by Graph Pad Prism 5.0. All data was expressed as mean ± standard error from at least 3 independent experiments. Differences between mean