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Zerumbone modulates CD1d expression and lipid antigen presentation pathway in breast cancer cells

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ABSTRACT

Natural Killer T (NKT) cells based cancer immunotherapy is an evolving area of cancer therapy, but tumors escape from this treatment modality by altering CD1d expression and its antigen presentation pathway. Here, we have studied the relation of CD1d expression in various breast cancer cell lines to their viability and progression. We observed a novel phenomenon that CD1d expression level increases with the progressive stage of the cancer. A small molecule, zerumbone (ZER) caused down-regulation of CD1d that was accompanied by breast cancer cell growth *in vitro*. The growth inhibitory effect of ZER against breast cancer cells was augmented by treatment with anti-CD1d mAb. This effect was mediated by G1-phase cell cycle arrest and apoptosis induction coupled with an increase in mitochondrial membrane depolarization. CD1d expression and cell proliferation were inhibited by both CD1d siRNA and ZER. The α-galactosylceramide, a ligand for CD1d, showed increased CD1d expression as well as cell proliferation which was opposite to the effects of ZER. This study shows that, CD1d overexpression is associated with the progressive stages of breast cancer and ZER could be an adjuvant to potentiate cancer immunotherapy.

1. Introduction

Breast cancer is the foremost diagnosed cancer and a leading cancer related fatality in females. Around 1.67 million new breast cancer cases and 0.52 million deaths were recorded among women worldwide which accounted for 25% estimated incidence rate and 15% cancer death rate of all cancers among women (Ferlay et al., 2015). The advances in recent years suggest that immunotherapy could be a reasonable approach as compared to other parallel treatments in the contemporary therapeutics (Gajewski et al., 2013). The distinctive characteristic feature of cancer immunotherapy lies in basic fundamentals of immune system which recognizes and differentiates normal and tumor cells by expression of their immunogenic markers (antigens) both qualitatively and quantitatively (V.I. Seledtsov AASaGVS, 2011). Therefore, further understanding of new edges of cancer immunotherapy might serve as an efficient treatment modality.

Natural Killer T (NKT) cells contribute to a significant defense against various kinds of self or foreign antigens through their immunoregulatory actions resulting in protective or harmful consequences in pathological conditions such as microbial infection, allergic disease, autoimmune disease and cancer (Brigl and Brenner, 2010; Cohen et al., 2009; Meyer et al., 2007; Novak and Lehuen, 2011; Tupin et al., 2007; Vivier et al., 2012). There are two subsets of NKT cells, one is the type I NKT, commonly called as invariant NKT (iNKT) cells which are in major population, and another is the type II NKT which are less in number. iNKT cells play a significant role in tumor immunity (Pilones et al., 2014). The interaction of NKT cell receptor (TCR), self or foreign lipid based antigens and CD1d in antigen presenting cells (APCs) execute their activity against tumor cells by releasing cytokines and activating other immune cells by forming a bridge between innate and adaptive immunity (Brennan et al., 2013; Rossjohn et al., 2012; Salio et al., 2014; Wolf et al., 2014). Significant role of CD1d in NKT cell development has been reported where CD1d1 knockout mice were developed with deficient numbers of NKT cells

Abbreviations: iNKT, invariant Natural Killer T cells; mAb, monoclonal antibody; APCs, antigen-presenting cells; α-GalCer, α-galactosylceramide; ZER, zerumbone; MTTP, microsomal triglyceride transfer protein; AgPP, antigen presentation pathway; MTT, 3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide; DMSO, dimethyl sulfoxide; DAPI, 4′,6-diamidino-2-phenylindole

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(Mendiratta et al., 1997). In systemic lupus erythematosus disease, the reduced number and function of iNKT cells, were found to be restored (both increased number as well as functional iNKT) with increasing CD1d population (Bosma et al., 2012). As previously reported, in case of primary prostate cancer there was optimum level of CD1d expression but due to nonfunctional NKT cells, cancer cells escaped the defense system. NKT mediated anti-tumor response was restored by exogenously supplying alpha-galactosylceramide (α-GalCer) and IL-12 in prostate cancer and dendritic cells (Nowak et al., 2010; Sohn et al., 2014).

In different cancers, including renal cell carcinoma, peripheral T cell lymphoma, chronic lymphocytic leukemia and UV induced skin cancer, the higher expression of CD1d promoted tumor cell proliferation (Chong et al., 2015; Bachy et al., 2016; Jadidi-Niaragh et al., 2012; Matsumura et al., 2005). Considering problems associated with NKT-dependent tumor treatments, herein we explored CD1d as a treatment target without the help of NKT cells in breast cancer cells. There is a report of sulforaphane being a phytochemical augmenting NK cells activity against prostate tumor in mice (Singh et al., 2009). We studied the role of CD1d in tumor immunity mediated by a phytochemical ZER, known to have anti-cancer activities in several cancers including breast cancer. This molecule is a monocyclic seisquiterpene isolated from the plant *Zingiber zerumbet* (Sehrawat et al., 2014). ZER was reported to modulate Th1 response and induce the secretion of IFN-γ in a mouse model of asthma (Shieh et al., 2015). Considering the importance of CD1d expression in cancer cells, we have explored how this molecule gets regulated in presence of ZER, α-GalCer and anti-CD1d mAb.

2. Materials and methods

2.1. Chemicals and reagents

Zerumbone (Sigma Aldrich, St Louis, MO), α-GalCer (Toronto Research Chemicals, North York, ON), LEAF™ purified anti-human CD1d monoclonal antibody, LEAF™ purified mouse IgG2b, κ isotype control, anti-mouse CD16/32 antibody (Biolegend, San Diego, CA), N2C3 CD1d antibody (GeneTex, Irvine, CA), CD1d siRNA (Qiagen, Germantown, MD), PNgaseF (Sigma Aldrich, St Louis, MO) were used for the experiments. Antibodies of CDK2, Bax, Bcl-2, Phospho-p44/42 MAPK (ERK 1/2) (Thr202/Tyr204), Phospho-Akt (Ser473), total PARP [Poly (ADP-ribose) polymerase], cleaved-PARP, horseradish peroxidase conjugated mice and rabbit secondary were procured from Cell Signaling Technology, Danvers, MA. Cyclin E antibody was purchased from Santa Cruz Biotechnology, Dallas, TX, whereas β-actin antibody was from Sigma-Aldrich. Bromophenol blue, EDTA, EGTA, JC-1 dye, triton X-100, NP-40, DMSO, MTT, disodium hydrogen phosphate (Na_2HPO_4) and monopotassium phosphate were obtained from Sigma Aldrich. Annexin-V-FITC apoptosis detection kit was obtained from BD Biosciences, San Jose, CA, nitrocellulose membrane from Amersham Biosciences, Piscataway, NJ and enhanced chemiluminescence were purchased from Merck Millipore, Billerica, MA. All the remaining chemicals used were of highest purity grade.

2.2. Cell culture and treatment combinations

MDA-MB-231, MDA-MB-468, MDA-MB-361, T-47D were maintained in RPMI 1640 media and whereas MCF-7 and MCF-10A cells were maintained in MEM (HiMedia, Mumbai, India), and MEGM (Clonetics, San Diego, CA) media respectively containing 10% fetal bovine serum (FBS, Invitrogen-Life Technologies, Grand Island, NY), and antibiotics. These cells were purchased from American Type Culture Collection (Manassas, VA) (Xiao et al., 2006). For MCF-7 cells extra supplement of 1% human recombinant insulin (Sigma Aldrich, St Louis, MO) was added in the media. The SUM-159 cell line was purchased from Asterand (Detroit, MI) and maintained in Ham's F-12 media supplemented with 5% FBS, 5 μg/ml insulin, 1 μg/ml hydrocortisone and 10 mM HEPES. DMSO was used to prepare ZER and α-GalCer stocks. Culture of each cell line was carried out in a 5% CO_2 incubator at 37 °C (Sehrawat et al., 2012). The concentrations of treatments of chemicals were ZER (20 and 40 μM), CD1d siRNA (500 ng/ml), α-GalCer (250 and 500 nM) and anti-CD1d mAb (5 μg/ml) throughout the experiments. The vehicle controls used in different assays were control siRNA for knock-down studies, isotype control for mAb blockade assays and DMSO for ZER as well as α-GalCer treatments.

2.3. MTT cell proliferation assay

MTT assay was carried out by seeding 1×10^4 cells in 96-well culture plate and maintained at 37 °C in incubator. Effect of CD1d siRNA (500 ng/ml) and ZER (20 μM) on cell proliferation of MDA-MB-231 cells were analyzed after 96 h with vehicle control (DMSO and control siRNA). The cell proliferation was analyzed in MDA-MB-231 cells treated with vehicle control (DMSO), 250 and 500 nM concentrations of α-GalCer for 24 h. Effect of ZER (20 and 40 μM), anti-CD1d mAb (5 μg/ml) and ZER (20 μM) + anti-CD1d mAb (5 μg/ml) combination was analyzed on cell proliferation of MCF-7 and MDA-MB-231 cells. DMSO and isotype controls were used as vehicle controls. The effect on cell proliferation was observed through measuring the absorbance at 570 nm in Synergy™ H1 Hybrid Multi-Mode Microplate Reader (Sabarwal et al., 2016).

2.4. Trypan blue assay for cell viability

Cells were plated in 12 well cell culture dishes and treatments were given at 70% confluency. The cell viability assay was used to study the effect of ZER (20 μM) and CD1d siRNA (500 ng/ml) treatment on MDA-MB-231 cells after 96 h. This experiment was done with vehicle controls such as DMSO and control siRNA. The assay was also used to analyze the effect of ZER (20 and 40 μM), anti-CD1d mAb (5 μg/ml) and ZER (20 μM) + anti-CD1d mAb (5 μg/ml) treatments on cell viability after 24 h time point. For this experiment DMSO and isotype control were used as vehicle control. After completion of incubation time, trypan blue dye was added to the harvested cells obtained after trypsinization. Then cells were counted using hemocytometer visually through the inverted microscope and scored as dead or alive (Vundru et al., 2013).

2.5. Mitochondrial membrane potential analysis by JC 1 assay (ΔΨm)

MDA-MB-231 cells (0.5×10^5) were seeded in 6-well tissue culture plates and treated with ZER and anti-CD1d mAb with their respective vehicle controls (DMSO and isotype control). After 24 h of treatment cells were collected by using trypsin and centrifuged in respective falcon tubes and at 500 × *g* for 7 min followed by PBS (1 ×) washing. The cell pellets were re-suspended in 2.5 ml JC-1 (5 mg/ml stock) dye at room temperature for 10 min. Further, the cells were washed twice by centrifuging with 400 μl PBS at 500 × *g* speed in centrifuge for 5 min. Finally, cells were re-suspended in 300 μl of PBS and minimum of 5000 events was recorded using flow cytometry (Sahu et al., 2014).

2.6. Analysis of cell cycle distribution

In a 6 well plate, 0.5×10^5 cells were seeded and incubated for overnight. The propidium iodide (PI) staining was performed to measure cell cycle phase arrest in MDA-MB-231 cells treated with ZER (20 and 40 μM), anti-CD1d mAb (5 μg/ml) and ZER (20 μM) + anti-CD1d mAb (5 μg/ml) for 24 h and analyzed through flow cytometry. Here DMSO and isotype control were used as vehicle controls. Total cells including both floating as well as attached cells were collected and washed with 1 × PBS and fixed at 4 °C for overnight with 70% ethanol. Then cells were incubated for 30 min with RNaseA (80 μg/ml) and PI (50 μg/ml) and flow cytometry was carried out in FACS Aria III (BD