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α-Lipoic acid inhibits the migration and invasion of breast cancer cells through inhibition of TGFβ signaling

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ABSTRACT

Aims: Invasion and metastasis are the main cause of mortality in breast cancer. Hence, novel therapeutic interventions with high specificity toward invasion and metastasis are necessary. α-Lipoic acid showed antiproliferative and cytotoxic effects on several cancers including breast cancer. However, the effect of lipoic acid on breast cancer metastasis remains unclear.

Main methods: In the present study, we examined the effects of lipoic acid on the migration and invasion of MDA-MB-231 and 4T1 breast cancer cells.

Key findings: Our data showed that lipoic acid effectively inhibited the colony forming ability of highly invasive MDA-MB-231 and 4T1 cells. Moreover, the nontoxic concentrations of lipoic acid significantly reduced the migration of breast cancer cells. Lipoic acid also inhibited the TGFβ-induced angiopoietin-like 4 (ANGPTL4) expression and reduced the activity of matrix metalloproteinase-9 (MMP-9), an enzyme involved in invasion and metastasis, in both the cell lines. The inhibition of cell migration by lipoic acid is accompanied by the downregulation of FAK, ERK1/2 and AKT phosphorylation, and inhibition of nuclear translocation of β-catenin.

Significance: Our data demonstrated that lipoic acid inhibited the migration and invasion of metastatic breast cancer cells at least in part through inhibiting ERK1/2 and AKT signaling. Thus, our findings show that the inhibition of TGFβ signaling is a potential mechanism for the anti-invasive effects of lipoic acid.

1. Introduction

Breast cancer is the second most common life-threatening diseases seen in women. The two main reasons for the high mortality rates associated with breast cancer and the leading causes of poor clinical outcomes are invasion and metastasis [1]. Metastasis of breast cancer is a complex event, involving several coordinated sequential steps such as intravasation, survival in circulation, extravasation into distant organ and angiogenesis [2]. Recent studies have identified transforming growth factor beta (TGFβ) as one of the factors produced abundantly by stromal cells that is essential for tumor cell metastasis [3,4]. Activation of TGFβ signaling promotes breast cancer metastasis by increasing cell proliferation, epithelial–mesenchymal transition (EMT), migration, invasion, colonization at distant organs and by inhibiting the immune cell function [3,5,6]. Treatment with TGFβ has been shown to cause the delocalization or downregulation of cell junction proteins such as ZO-1, E-cadherin, β-catenin, and promotes cytoskeleton reorganization by forming stress fiber assembly and by myosin light chain phosphorylation [5,7]. The activation of TGFβ/Smad pathway induces breast cancer cell invasion by increasing the expression of matrix metalloproteinase 2 and 9 (MMP-2 and MMP-9) [8]. Therefore, a drug which can suppress the TGFβ signaling can be a promising agent for blocking breast cancer metastasis.

α-Lipoic acid, also known as thiotic acid, occurs naturally as a prosthetic group in various mitochondrial enzymes such as pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched-chain α-keto acid dehydrogenase, and glycine decarboxylase complex, and plays a fundamental role in metabolism [9]. Several studies have confirmed the beneficial effect of lipoic acid in the therapy of many diseases, including atherosclerosis, diabetes, neurodegeneration, arthritis, autoimmune diseases, liver damage and cancer [10,11]. Previous studies show that lipoic acid has a cytotoxic and antiproliferative effect on various cancers including breast cancer, leukemia, colon cancer and bladder cancer [12–15]. Lipoic acid inhibits the proliferation of breast cancer cells, MCF-7 and MDA-MB-231, and induces apoptosis in these cells [12–16]. Lipoic acid treatment also reduced the activity of MMP-2 and MMP-9 in

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MDA-MB-231 cells [17]. In thyroid cancer cells, lipoic acid inhibited the EMT, migration and invasion through suppression of TGFβ production in cells with activated MAPK signaling [18]. However, the effects of lipoic acid on the migration and invasion of breast cancer cells are unknown. We hypothesize that lipoic acid might inhibit the migration of breast cancer cells by inhibiting the TGFβ signaling. We have evaluated our hypothesis by studying the effect of lipoic acid on TGFβ-induced migration and invasion of MDA-MB-231 and 4T1 cells. We have also studied the effect of lipoic acid on the expression of MMP-9 and other markers of cancer cell invasion.

2. Materials and methods

2.1. Chemicals and reagents

DL-α-Lipoic acid was purchased from HiMedia laboratories (Mumbai, India). TGFβ1 was purchased from Abcam (Cambridge, MA, USA). PCR primers were purchased from Eurofins genomics (Bangalore, India). Primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and Cell Signaling Technology, Inc. (Danvers, MA, USA). Fetal bovine serum, RPMI and other cell culture reagents were purchased from PAN-Biotech (Germany). All other chemicals and reagents used were of analytical grade.

2.2. Cell culture and treatments

MDA-MB-231 (human) and 4T1 (mouse) breast cancer cell lines were obtained from National Centre for Cell Science, Pune, India. MDA-MB-231 and 4TI cell lines are triple negative i.e. human epidermal growth factor receptor 2 (HER-2) neu-negative, estrogen receptor (ER-negative) and progesterone receptor (PR-negative). MDA-MB-231 and 4T1 cells were maintained in RPMI 1640, supplemented with penicillin (100 U/ml), streptomycin (100 U/ml) and 10% fetal bovine serum at 37 °C in 5% CO_2–95% air atmosphere in a humidified incubator. The cells were subcultured every 5 to 6 days. Lipoic acid was dissolved in 0.1 N NaOH and neutralized with HCl and added to the culture media. Cells were treated with lipoic acid at 40–50% confluence and harvested at specified times. TGFβ1 was reconstituted in 10 mM citric acid, pH 3.0 to a concentration of 50 μg/ml and stored at −20 °C.

2.3. Cell proliferation

MTT assay was used to detect the viable proliferating cells. Briefly, 8000 cells were seeded in 96-well plates and treated with lipoic acid at various concentrations (0.25 to 2.5 mM), for 24, 48 and 72 h. After treatment, MTT reagent was added and incubated for 4 h in dark for formazan crystal formation. Then, the crystals were dissolved and the absorbance was measured at 570 nm in a microplate reader.

2.4. Colony formation assay

For anchorage-dependent colony formation assay, 500 cells were seeded in 6 well plates and incubated for 24 h. Then, the cells were treated with lipoic acid (0.25 to 2.5 mM) and incubated till colonies were formed (10 days). The culture media was replaced every 3 days. The colonies were fixed and stained with crystal violet for 1 h at room temperature. The wells were washed with water, dried overnight at room temperature and the images were taken. Finally, the stain was dissolved in 10% glacial acetic acid and the absorbance of the released dye was measured at 590 nm.

Anchorage-independent growth was assessed as previously described [19]. Briefly, in 6 well plates, base agar was made by 0.75% agarose in culture media. Top agarose overlay was made fresh by mixing 0.66% molten agarose with 2× culture media containing 2000 cells/well, and then gently overlaid over the base agar. The plates were incubated for 21 days and the medium was replaced twice a week. The colonies were stained with crystal violet and counted visually.

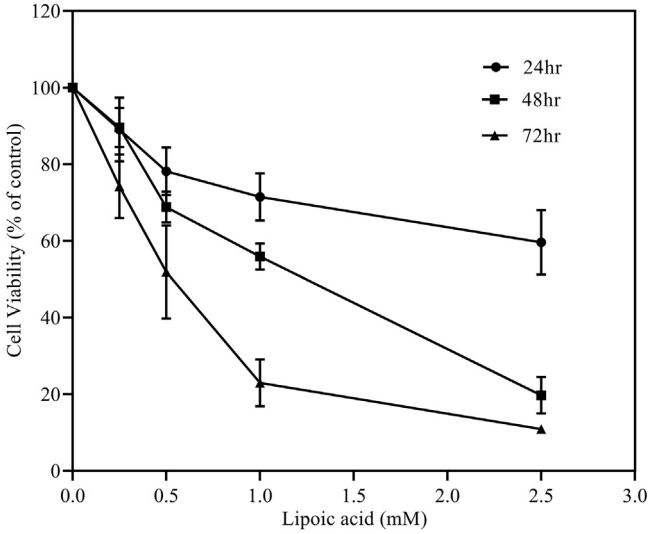

Fig. 1. Lipoic acid inhibits the proliferation of MDA-MB-231 cells. MDA-MB-231 cells were plated in 96 well cell culture plates and treated with lipoic acid. After 24, 48 and 72 h, the MTT assay was carried out as described in materials and methods. The cell viability is directly proportional to the amount of formazan crystal formation.

2.5. Cell migration assay

The MDA-MB-231 and 4T1 cells were cultured in 6 well plates to near confluence (90%). With the help of 200 μl micropipette tip, a wound is created in the cells and rinsed with PBS. The images of the scratched area were taken in an inverted microscope. Then the cells were treated with lipoic acid (0.25 to 2.5 mM) and/or TGFβ1. The images were recorded at different time points and analyzed to evaluate the migration of cells into the scratched area.

2.6. Measurement of MMPs activity by gelatin zymography

MDA-MB-231 and 4T1 cells were pre-treated with 0.5 and 1 mM lipoic acid for 48 h. Then the media was exchanged with serum-free media containing lipoic acid and/or 200 pM TGFβ1. After 24 h, the media was collected and concentrated. The protein content was estimated and 40 μg of protein samples were separated under non-reducing conditions on polyacrylamide gels containing 0.1% gelatin. After electrophoresis, gels were washed with renaturation buffer (2.5% Triton X-100) for 1 h, and subsequently incubated in 50 mM Tris-HCl, pH 7.5, containing 10 mM $CaCl_2$, 1 μM $ZnCl_2$ and 200 mM NaCl for 16 h at 37 °C with constant rocking. Gels were stained with 0.1% Coomassie brilliant blue R-250 and partially destained to get the clear zones within the blue background which indicate the proteolytic activity of MMPs.

2.7. Quantitative real-time PCR

MDA-MB-231 and 4T1 cells were treated with 0.5 and 1 mM lipoic acid for 48 h with serum containing media, followed by 4 h treatment with TGFβ1 (200 pM) and/or lipoic acid in serum-free medium. Then, the total RNA was extracted from the cells using TRIzol total RNA isolation reagent as per the manufacturer's protocol. cDNA was synthesized from 1 μg of total RNA using Fermentas cDNA synthesis kit (Thermo Scientific, PA, USA). Real-time PCR was performed to check angiopoietin-like 4 (ANGPTL4) expression using SYBR green assay kit. The primers sequences used are listed in (Supplementary Table 1). The GAPDH expression was used as internal control.