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Effects of chemotherapy agents on Sphingosine-1-Phosphate receptors expression in MCF-7 mammary cancer cells

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ABSTRACT

Sphingosine-1-phosphate (S1P) is a potent bioactive sphingolipid involved in the regulation of cell proliferation and cancer progression. Increased expression of S1P receptors has been detected in advanced breast tumours with poor prognosis suggesting that S1P receptors might control tumour response to chemotherapy. However, it remains unclear how the levels of S1P receptor expression are influenced by chemotherapy agents. Western immunoblotting, PCR analysis and fluorescent microscopy techniques were used in this study to analyze expression patterns of S1P receptors 2 and 3 (S1P2/S1P3) in MCF-7 breast adenocarcinoma cells treated by Tamoxifen (TAM) and/or Medroxyprogesterone acetate (MPA). We found that TAM/MPA induce downregulation of S1P3 receptors, but stimulate expression of S1P2. According to cell viability and caspase activity analyses, as expected, TAM activated apoptosis. We also detected TAM/MPA-induced autophagy marked by formation of macroautophagosomes and increased level of Beclin 1. Combined application of TAM and MPA resulted in synergistic apoptosis- and autophagy-stimulating effects. Assessed by fluorescent microscopy with autophagosome marker LAMP-2, changes in S1P receptor expression coincided with activation of autophagy, suggestively, directing breast cancer cells towards death. Further studies are warranted to explore the utility of manipulation of S1P2 and S1P3 receptor expression as a novel treatment approach.

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1. Introduction

Sphingolipids regulate several essential biological functions including maintenance of structural stability of cellular membranes and transduction of signals to and from major biomolecular effectors, such as hormones and growth factors receptors. One of the most important and ubiquitously expressed bioactive sphingolipid metabolizing enzymes is sphingosine kinase (SphK) [1]. The tumour promoting role of SphK and its phosphorylation product, Sphingosine-1-phosphate (S1P), has been demonstrated in various cancer tissues [2]. Majority of cells, including malignant cells, can sense the level of S1P in blood and lymph using plasma membrane S1Pn (where n = 1–5) receptors. S1P receptors are differentially coupled to heterotrimeric G-proteins, which can initiate a multitude of downstream signalling cascades [3]. S1P1, S1P2, and S1P3 receptors are widely expressed [4] and considered to be crucial regulators of cancer cell growth and survival via activation of key pathway clusters such as Ras/Erk and PI3 K/Akt [5,6].

Positive correlations between estrogen-stimulated proliferation and activation of SphK followed by increased intracellular S1P have been demonstrated in breast cancer cells [7,8]. S1P and its receptors are also involved in transactivation of growth factor signalling mechanisms [9] and delay degradation of epidermal growth factor receptors (EGFR) [10]. Tamoxifen (TAM)-resistant breast cancer MCF-7 cells demonstrate increased level of SphK expression and activity *in vitro* [11], raising the possibility that S1P receptor expression might be influenced by TAM treatment. Further, S1P receptors expression levels are higher in estrogen receptor (ER) positive advanced breast tumours [12]. Notably, the higher level of S1P receptor expression is associated with shorter recurrence time in patients treated for at least 5 years with adjuvant TAM [12]. These findings suggest that TAM effects might be interconnected with expression of S1P receptors. However, experimental confirmation of this has remained unreported. As TAM and medroxy-progesterone acetate (MPA) are widely used for the endocrine treatment of breast cancers [13,14], in this study we assessed the level of S1P receptors after TAM and/or MPA

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treatment in chemotherapy-responsive ER-positive MCF-7 breast cancer cells.

2. Materials and methods

In this study, we determined the effect of various doses of TAM and/or MPA on S1P receptor (S1P3 and S1P2) expression in the MCF-7 breast adenocarcinoma cell line *in vitro* during 7 days of treatment. To confirm that chemotherapy agents (TAM and MPA) induce significant biological effects (apoptosis and decreased cell viability) in MCF-7 cells, we used caspase 2 activation and MTS cell viability assays. Markers of autophagy were assessed using brightfield and confocal microscopy, along with western blotting analysis of Beclin-1 expression. We also used immunofluorescent confocal microscopy to visualize any detected changes in S1P receptors expression levels and localization in cancer cells undergoing TAM/MPA-induced autophagy.

2.1. Cells, culture medium, chemicals, and bright field microscopy

Human breast cancer MCF-7 cells were obtained from the American Type Culture Collection (ATCC HTB-22) and cultured in phenol red-free Dulbecco's modified Eagle's medium (CSL Biosciences, Parkville, Australia) supplemented with 10% heat inactivated fetal bovine serum (Invitrogen), 2 mM L-glutamine (Invitrogen), nonessential amino acids (1:100) (GIBCO), penicillin (100 U/ml), and streptomycin (100 μg/ml) (GIBCO) as described [9]. The complete medium was filter-sterilized (0.2 μm pore-size Nalgene filter units). Tamoxifen (T5648), Medroxy progesterone acetate (M1629), Rapamycin (R8781) (an inducer of autophagy [15]) were purchased from Sigma-Aldrich (USA).

Bright field microscopy (20×) was used to monitor formation of autophagic vacuoles daily. After 7 days treatment with various doses of TAM and MPA (from 1 nM to 10 μM) every treated well within 6 well plates was photographed at randomly chosen areas. Cells, with visible vacuoles were counted per image in each experimental group; percentage of vacuolated cells was estimated. Mean percentage value was derived from 3 independent experiments.

2.2. Western immunoblotting analysis

To assess protein expression levels of S1P2/S1P3 receptors and autophagy marker Beklin-1 in MCF-7 breast cancer cells treated *in vitro* by TAM and/or MPA we used western blotting. The background endogenous expression levels of S1P2/3 receptors were previously shown in MCF-7 cells in our previous experiments [9–11]. Cells were harvested and lysed in buffer containing protease inhibitors cocktail (Sigma-Aldrich). Aliquots of cell lysates with equal amounts of total protein (50 μg) were resolved on 12% SDS-polyacrylamide gel and transferred to Hybond-P membranes (Amersham). The membranes were then probed with the appropriate primary antibodies, according to manufacturer's standard method. Horseradish peroxidase-conjugated secondary antibody (BioRad Laboratories, USA) and the enhanced chemiluminescence protein band detection system (BioRad Laboratories, USA) were used. Protein densitometry was performed on a mode imager (LAS 4000, GE Healthcare Life Sciences) and images were analysed using Carestream Molecular Imaging (Carestream Health Australia Pty Ltd). S1P3 receptor antibodies were purchased from Sapphire Biosciences Pty. Ltd. Beclin-1 antibodies were purchased from Epitomics Inc. (USA). β-actin and S1P2 receptor antibodies were from Cayman Chemicals (USA). S1P3 antibodies were used in 1:500 dilution and S1P2–1:1000 a recommended by manufacturers.

2.3. Cell viability assay

Trypsin (Invitrogen) was used to harvest 90% confluent cells. Cells were then counted using TC10™ Automated Cell Counter (Bio-Rad). Cells (2×10^4 cells/100 μl/well) were seeded in eight replicates into 96-well plates (Greiner Bio-One) and incubated at 37 °C in 5% CO_2 overnight to allow cell attachment. The cells were then treated for 7 days with vehicle control (0.2% ethanol) or with serial dilutions of TAM (1 nM–10 μM), MPA (1 nM–10 μM) or TAM (1 nM–10 μM) + MPA (10 nM) in phenol-red free media for 7 days. To assess cell viability in the presence of TAM and MPA we used the CellTiter 96 Aqueous Proliferation Assay (Promega Corporation, Sydney, Australia) as described in the manufacturer's protocols using a Bio-Rad Microplate reader (Model 680; Bio-Rad Laboratories, CA, USA). Cell numbers were calculated based on calibration curves and normalized to seeding density.

2.4. Caspase 2 assay

Lack of normal expression of caspase-3 mRNA and protein was reported in MCF-7 cells [16], therefore activation of apoptosis in MCF-7 cells was assessed using caspase-2 colorimetric assay (Calbiochem®, Germany). After 7 day treatment with various doses of TAM and/or MPA (as described above), 2×10^6 cells grown in 6 well plates were lysed in chilled lysis buffer, centrifuged at 10,000×g for 2 min. The collected and diluted (200 μg protein in 50 μl cell lysis buffer) supernatants were mixed with reaction buffer containing 10 mM DTT, and incubated with VDVAD-*p*NA substrate at 37 °C for 2 h according to the manufacturer's protocol. Sodium Butyrate (NaB) (1 mM) was used as apoptosis-inducing positive control reagent. Caspase-2 activity in the presence of NaB was considered as 100%. Absorbance was measured at 405 nm using DTX 880 Multimode Detector (Beckman Coulter) microtiter plate reader.

2.5. Real-time RT-PCR

PCR was used to evaluate the level of S1P receptor mRNA. Total RNA was isolated with TRIzol reagent (Invitrogen). RNA was reverse transcribed using the Quantiscript®RT kit for reverse transcription (Qiagen). For real-time PCR, primers were purchased from Geneworks (South Australia). The sequences of PCR primers used were for S1P3 receptor: forward 5′TCAGCCTGTCTCCCACGGTC3′ and reverse 5′AACGGCTGCTGGACTTCACCA3′; for S1P2 receptor: forward 5′CCGAAACAGCAAGTTCCACT3′ and reverse 5′CCAGGAGGCTGAAGA-CAGAG3′; for 18S rRNA: forward 5′CCGCAGCTAGGAATAATGGA3′ and reverse 5′GTCGGCATCGTTTATGGTC3′. PCR amplification was performed using Quantitect® SYBR Green mastermix (Qiagen) on a Rotor Gene 6000 (Corbett Research). Quantitative real-time RT-PCR analysis was then performed using Q-Gene software [17]. The mRNA of interest was normalized to 18SrRNA.

2.6. Immunofluorescent confocal microscopy

S1P receptor expression and cellular localization were also visualized using confocal microscopy. MCF-7 cells were seeded onto Fibronectin (Invitrogen) coated glass slides (Lab-Tech) and cultured with 100 nM TAM and/or MPA for 7 days. After stimulation, cells were fixed with 4% paraformaldehyde and permeabilised in 0.1% Triton X-100 as described previously [10]. Cells on slides were incubated with primary antibody S1P2, or S1P3 and LAMP-2 (Abcam) for 2 h at 37 °C, washed 3 times for 5 min each with PBS, and incubated for 1 h at room temperature with secondary

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