Journal of Trace Elements in Medicine and Biology 28 (2014) 486–491

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.de/jtemb

X. ISTERH CONFERENCE

Effects of selenium compounds on proliferation and epigenetic marks of breast cancer cells

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ARTICLE INFO

Keywords:
MCF-7 breast cancer cells
Epigenetic marks
Apoptosis
Selenium

ABSTRACT

Breast cancer is a global public health problem and the most frequent cause of cancer death among women. Mammary carcinogenesis is driven not only by genetic alterations but also by epigenetic disturbances. Because epigenetic marks are potentially reversible they represent promising molecular targets for breast cancer prevention interventions. Selenium is a promising anti-breast cancer trace element that has shown the modulation of DNA methylation and histone post-translational modifications in other malignancies. This study aimed to evaluate the effects of selenium compounds [methylseleninic acid (MSA) and selenite] on cell proliferation and death, expression of the tumor suppressor gene RASSF1A and epigenetic marks in MCF-7 human breast adenocarcinoma cells. Treatment with MSA or selenite markedly inhibited ($P<0.05$) in a dose-dependent manner the proliferation of MCF-7 cells. MSA induced ($P<0.05$) G2/M cell arrest while selenite presented the opposite effect. Regarding cell death induction, MSA acted mainly by inducing apoptosis ($P<0.05$), while selenite only induced necrosis ($P<0.05$). Furthermore selenite, but not MSA, markedly induced ($P<0.05$) cytotoxicity and increased ($P<0.05$) RASSF1A expression. Both selenium compounds inhibited ($P<0.05$) DNMT1 expression. MSA decreased ($P<0.05$) H3K9me3 and increased ($P<0.05$) H4K16ac, while selenite decreased ($P<0.05$) this latter histone mark. To the best of our knowledge this is the first report showing that selenite and MSA modulate epigenetic marks specifically in breast cancer cells. Our data reinforce the anti-breast cancer potential of selenium that is dependent on its chemical form. Furthermore the data show that epigenetic mechanisms represent relevant molecular targets involved in selenium inhibitory effects in breast cancer cells.

Introduction

Breast cancer remains the most common malignancy and the second leading cause of death in women [1]. Mammary carcinogenesis is driven not only by genetic alterations but also by epigenetic disturbances [2]. Epigenetics involves altered gene expression without any change of gene sequences [2]. Global DNA hypomethylation and hypermethylation of promotor regions of tumor suppressor genes mediated by DNA methyltransferases (DNMTs) and histones post-translational onco-modifications comprise frequent epigenetic deregulation in breast cancer [3]. Acetylation and methylation of histones can define the chromosomal structure and gene expression state of breast tumor-related genes [4]. The tumor suppressor gene Ras-association domain family 1A (RASSF1A) is frequently silenced in breast cancer by hypermethylation of its promoter region [5].

Because epigenetic marks are potentially reversible they represent promising molecular targets for breast cancer prevention interventions [6,7]. Evidence from animal and cell culture experiments shows that the anticancer effects of selenium may involve interference with DNA methylation and histone modifications [8,9]. The chemical form of the essential element can further influence epigenetic modulation. Selenite but not seleno-*DL*-methionine restored expression of the tumor suppressor gene *GSTP1* by inhibition of HDAC activity and downregulation of DNMT expression in LNCaP prostate cancer cells [9].

Selenite and methylseleninic acid (MSA) represent promising selenium compounds with anti-breast cancer potential [10]. MSA is a stable selenium-containing organic compound with a single methyl group that represents a stripped down version of Se-methylselenocysteine without the amino acid moiety; while selenite, an inorganic selenium form, can be obtained directly

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http://dx.doi.org/10.1016/j.jtemb.2014.06.017
0946-672X/

from the diet [11]. Few studies have addressed epigenetic modulation by selenium in the context of its cancer preventive actions. Despite increasing evidence showing that selenium anti-cancer effects involve multiple mechanisms including antioxidant defense and cell proliferation inhibition, no study examined the association of selenium epigenetic modulation specifically in breast cancer. Thus we aimed to investigate the effects of MSA and selenite on proliferation, apoptosis, RASSF1A gene expression and epigenetic marks in MCF-7 (estrogen-receptor positive) breast cancer cells.

Materials and methods

Cell culture and selenium treatments

MCF-7 (ATCC, USA) cells were cultured as a monolayer in Dulbecco's modified Eagle's medium (DMEM; GIBCO, USA) supplemented with FBS (10%, v/v) (GIBCO, USA). Those cells were maintained in a humidified incubator chamber with an atmosphere of 5% of CO_2 at 37 °C until logarithmic growth (~80% of confluence) was established (3–4 days). Selenium compounds, MSA or selenite (Sigma Aldrich, USA), were dissolved in DMEM culture medium (GIBCO, USA), producing 2 mmol/L stock solution and stored at −20 °C. Immediately before each experiment, dilutions were made and added to the medium to achieve the desired final concentrations of MSA and sodium selenite.

Proliferation assay

For the cell proliferation assay, MCF-7 cells (2×10^5 cells/mL) were seeded on 96-well microplates and after a 24 h attachment period they were treated with a series of concentrations of MSA (1, 1.3, 1.6 and 2 μmol/L) or sodium selenite (1, 2, 5, 8, 10 and 20 μmol/L). Cells without MSA or selenite treatments served as controls. After 24, 48, 72, 96, 120 and 144 h incubation periods, cells were stained with 0.1% w/v crystal violet for 10 min at room temperature and absorbance measured at 570 nm.

Cytotoxicity assay

MCF-7 cells (5×10^5 cells/mL) were seeded for 24 h on a 6-well plate and treated with MSA (1.6 or 2 μmol/L; 96 h) or selenite (8 or 10 μmol/L; 120 h). Cells were harvested with trypsin–EDTA, incubated with propidium iodide (PI; 20 μg/mL) (Sigma Chemical, USA) in saline buffer for 5 min and evaluated on a FACS CANTO II flow cytometer (BD Biosciences, USA), using FlowJo vs 8.8.6 software. Ten thousand events were analyzed per experiment and the assay was done in triplicate. Fluorescence was measured on PEA channel (488/695 nm).

Cell cycle analysis

MCF-7 cells were treated with MSA and selenite as described in the cytotoxicity assay, trypsinized and fixed for 30 min on ice in 75% ice-cold methanol. The samples were incubated overnight at 4 °C, washed with PBS and resuspended in 200 μL of a solution of PBS containing PI (10 μg/mL) and RNase (15 mg/mL). The samples were then incubated for 60 min on ice and analyzed on a FACS CANTO II flow cytometer (BD Biosciences, USA). The assay was done in triplicate.

Apoptosis analysis

MCF-7 cells were stained with annexin V coupled with fluorescein isothiocyanate (FITC) and PI using an Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, USA) to measure phosphatidylserine (PS) externalization, in order to distinguish the apoptotic, necrotic and viable cell populations. MCF-7 cells (1×10^5 cells/mL) were seeded for 24 h on a 6-well plate and treated with MSA (1.6 or 2 μmol/L; 96 h) or selenite (8 or 10 μmol/L; 120 h). Cells were collected and resuspended in binding buffer (10 mmol/L HEPES/NaOH, 140 mmol/L NaCl and 2.5 mmol/L $CaCl_2$). Cells were incubated in the dark for 15 min at room temperature with Annexin V-FITC (1 mg/mL), followed by the addition of PI solution (100 μg/mL) and analyzed on a FACS CANTO II flow cytometer (BD Biosciences, USA), using FlowJo vs 8.8.6 software. The assay was done in triplicate.

Western blot analysis

MCF-7 cells (8×10^6 cells/mL) were seeded for 24 h in 100 mm cell culture dishes and treated with MSA (1.6 or 2 μmol/L; 96 h) or selenite (8 or 10 μmol/L; 120 h). Cells were harvested and histone and total protein were extracted as previously described by Jeong et al. [12] and Andrade et al. [13]. Extracts containing 30 μg of proteins were separated on 15% SDS-PAGE and transferred to PVDF membranes. The blots were incubated at 4 °C overnight with mouse primary antibodies at the following concentrations: anti-H3K9ac, 1:5000; anti-H3K9me3, 1:5000; anti-H4K16ac, 1:5000; anti-DNMT1, 1:5000; anti-H1 1:5000 (Upstate Biotechnology, USA); anti-β-actin, 1:5000 (Santa Cruz Biotechnology, USA). They were then washed with PBST (0.1% Tween-20 in PBS buffer) and incubated with horseradish peroxidase conjugated goat anti-rabbit antibody (1:10,000; GE Healthcare) for 1 h. Protein bands detected at 11, 17, 30 and 200 kDa corresponded to the expected molecular weight of histone H4, H3, H1 and DNMT1 protein, respectively. H1 and β-actin were used as internal control for normalization of histones and proteins, respectively. Membranes were developed using the ECL Advance Chemiluminescence kit (GE Healthcare, USA), and bands were revealed with the ImageQuant 400 capture imaging system (GE Healthcare, USA).

Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was obtained from MCF-7 cells with the RNAspin Mini Kit (GE Healthcare, USA), and cDNA was synthesized using 1 μg of total RNA using SuperScript™ First-Strand Synthesis System (Invitrogen, USA) for RT-PCR, according to manufacturer instructions. Quantification of mRNA expression of RASSF1A gene was performed in triplicate by qRT-PCR using the TaqMan amplification system (Applied Biosystems, USA). Assay on Demand probe (Applied Biosystems, USA) was used for RASSF1a (Hs00200394_m1) qRT-PCR analysis. GAPDH was used to normalize qRT-PCR data. mRNA levels were quantified using the ABI Prism 7000 Sequence Detection System. The thermal cycling conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 50 cycles of 96 °C for 15 s and 60 °C for 1 min. Amplicons were quantified using the 2(-Delta Delta C(T)) method according to Livak and Schmittgen [14].

Promoter methylation-specific PCR analysis

Genomic DNA was obtained from MCF-7 cells using phenol–chloroform and isoamyl alcohol method, stored at −80 °C and quantified by spectrophotometry (Nanodrop1000 Thermo-Scientific). DNA (2 μg) was subjected to bisulfite modification to convert all unmethylated cytosines to uracils [15]. The modified DNA was amplified with specific primers that were constructed for RASSF1A promoter gene region. Unmethylated primers: 5′-GGT TGT ATT TGG TTG GAG TG-3′ (sense); 5′-CTA CAA ACC TTT ACA CAC AAC A-3′ (anti-sense). Methylated primers: 5′-GTT GGT ATT CGT TGG GCG C-3′ (sense); 5′-GCA CCA CGT ATA CGT AAC G -3′ (anti-sense).