Inhibitory Effect of Selenium on Esophagus Cancer Cells and the Related Mechanism

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Summary  Selenium has been associated with many malignant tumors including esophagus cancer (EC). In current study, we examined the effects of three types of selenium, sodium selenite (SSE), methylseleninic acid (MSA) and methylselenocysteine (MSC) on EC cell line Eca109. Here, selenium attenuated cell viability and increased cell apoptosis, especially in MSC, when compared with control group (p<0.05). Meanwhile, MSC and MSA, but no SSE, arrested cell cycle in G0/G1 phase (p<0.05). Mechanistically, FAL1 and PTEN were found to participate in regulating cell cycle and cell apoptosis process by decreasing cyclinD1, CDK2, and promoting caspase-3, caspase-8. In addition, we found that cyclinD1, CDK2 were significantly downregulated by MSA and MSC, while caspase-3, caspase-8 were dramatically upregulated by SSE (p<0.05). Based on these results, we concluded that MSC and MSA inhibit the viability of Eca109 mainly through reducing cell proliferation, while SSE by promoting apoptosis.

Key Words  sodium selenite, methylseleninic acid, methylselenocysteine, cell proliferation, apoptosis

Esophagus cancer (EC) is one of the most serious and prevalent cancer worldwide (1). There are two main subtypes of EC, esophageal squamous-cell carcinoma and esophageal adenocarcinoma, the former one contributes for almost 90% of EC cases (2). According to previous epidemiological and clinical studies, long time smoking, alcohol, and obesity may be associated with EC (2). Radiotherapy and chemotherapy have been used to treat EC for years, but a large proportion of newly diagnosed EC patients still die because of its rapid progression. Therefore, investigating the pathological mechanism of EC and finding out associated molecular and genetic variation in the earlier stages will be of great help for timely diagnosis and therapy for EC.

Selenium (Se) is an essential and unique trace element that plays a crucial role in cancer prevention. The underlying anti-neoplastic mechanism of selenium has been provided as anti-oxidation (3), regulation of cell cycle (4), and apoptosis (5). However, the definite role of selenium plays in EC cells still unknown. Therefore, to further investigate the potential interfere of selenium for EC cells will be valuable to elucidate the underlying biomarkers and provide therapeutic targets of EC.

Hundreds of gene and protein expression changes during the progression of EC (6), and genetic changes plays a critical role in modulating cell viability, proliferation and invasion (7). Long non-coding RNA (lncRNA) is one subtype of no protein-coding RNA (ncRNA) with longer than 200 nucleotides (nts) (8). Increasing studies proved that IncRNA participate in series of molecules biological processes by forming complexes including RNA-RNA, RNA-DNA, or RNA-protein, and these complexes play important role in cell physiology and pathology which lead to many cancers, including EC (9). Focally amplified lncRNA on chromosome 1 (FAL1), a family number of IncRNA, has been confirmed as an oncogene in different human cancers (10). For example, increased expression of FAL1 in thyroid cancer cells regulated the expression of cyclinD1, p21, and CDKN1A which are cell cycle-related proteins (11), cancer cell proliferation can be retarded by knockdown FAL1 through FAL1-spacial small interfering RNA (siRNA) (12).

In present study, we investigated the biological function of selenium on EC cell lines Eca109, also further explored FAL1 and cell viability-related gene and protein expression induced by selenium. The results could provide new evidence for EC diagnosis and therapy.

MATERIALS AND METHODS

Cells and reagents.  Cell line (Eca109) was bought from Cell Bank of the Chinese Academy of Science (Shanghai, China). MTT, DMSO, sodium selenite (SSE; purity≥95%), methylselenic acid (MSA; purity ≥95%) and Se-methylselenocysteine (MSC; purity ≥95%) were obtained from Sigma (St. Louis, USA). Dulbecco’s modified eagle medium (DMEM), fetal bovine serum (FBS), total RNA extraction kit (TRIzol), Annexin V-FITC apoptosis detection kit and enhanced chemiluminescence (ECL) kit were bought from Invitrogen...
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Transcript assay kit, Real Time PCR assay kit, Polyclonal CDK2, cyclinD1, caspase-3, caspase-8, PTEN and GAPDH antibodies were obtained from Santa Cruz Biotechnology (California, USA).

Cell culture and treatment. Cells were maintained in an incubator at 37°C with 5% CO₂, supplemented with DMEM medium containing 10% fetal bovine serum, 100 μg/mL penicillin G, and 100 μg/mL streptomycin sulfate, and medium was changed every 3 d.

Four groups (Control, SSE, MSA, MSC) were designed for MTT experiment to evaluate the adverse effects of selenium on cell viability. Cells were incubated with different concentrations of selenium for 24, 48, and 72 h, and the specific concentrations of the selenium were as follows: SSE (10, 20, 40 μmol/L), MSA (10, 20, 40 μmol/L), MSC (100, 200, 400 μmol/L), according to previous study (13) and our preliminary result.

Following MTT assay result, the appropriate experiment conditions selected for the subsequent experiments (flow cytometry, cell apoptosis assay, RT-PCR, and Western-blot) were determined at 48 h incubation. 40 μmol/L of SSE, 20 μmol/L of MSA, 100 μmol/L of MSC, for the similar cell viability.

MTT assay. MTT assay was curried out to assess cell viability. Briefly, 3,000 cells per well were seeded into 96-well plates and incubated for 24, 48, and 72 h with different concentration of selenium. The MTT (0.5 mg/mL) was added to the cells at 37°C. After incubating with MTT for 4 h, DMSO was added in the dark and co-culture with cells for another 10 min. Finally, OD

Table 1. Primer sequences used in real-time PCR.

| Gene name | Forward sequence | Reverse sequence |
|-----------|------------------|------------------|
| Cyclind1  | 5'-GCTGCTCTGTGGAACGCAAGC-3' | 5'-CACAGAGGCACGAAAGTTC-3' |
| CDK2      | 5'-TTTGCTGAGTGGTACGGCAGCTTGACGCAACC-3' | 5'-AGGGTCTGCTGTGCTCAGACATTTGGC-3' |
| Caspase-3 | 5'-ACCGATGTCATCAGCAGCTA-3' | 5'-AGGTCCGCTGTGCTTCACAAAAA-3' |
| Caspase-8 | 5'-TACTACCGAAAATCCGACC-3' | 5'-GTTACGATAGGTTGCTGAC-3' |
| PTEN      | 5'-TGGATTTGACTTTAGCTTGACCT-3' | 5'-GGTGGTTATGCTTCTAAAGG-3' |
| FAL-1     | 5'-TGGATTTGACTTTAGCTTGACCT-3' | 5'-GGTGGTTATGCTTCTAAAGG-3' |
| GAPDH     | 5'-ACCACCTCTCCACCTTTGGA-3' | 5'-CTGTTCGCTAGCCAAATTGT-3' |

Fig. 1. Effect of selenium on viability of EC cells. Cells were treated with 0 (control), 10 (low), 20 (medium), 40 (high) μmol/L of SSE and 0 (control), 10 (low), 20 (medium), 40 (high) μmol/L of MSA or 0 (control), 100 (low), 200 (medium), 400 (high) μmol/L of MSC for 24 (A), 48 (B) or 72 (C) h. One-way ANOVA was used for comparison between treatments for each concentration. Data are expressed as mean±SD. Different characters in each set (24, 48 or 72 h) mean significant differences (p<0.05).
value was recorded at 570 nm.

Cell cycle analysis. Cell cycle of Eca109 was detected using flow cytometric analysis. After co-cultured with different selenium for 48 h, cells were collected and fixed with 70% ethanol for 24 h. Then cells were stained with 400 μL propidium iodide (PI). Cell cycle distribution was analyzed at the wavelength of 488 nm.

Cell apoptosis assay. Cells were incubated with different selenium, 48 h later; flow cytometric assay was used to determine apoptosis through an Annexin V-FITC apoptosis detection kit following the manufacturer’s protocol. The following equation was used to calculate apoptosis rate: Apoptosis rate = (UR + LR) / (UR + LR + LL) × 100%. UR stand for the late apoptosis cells in upper right area of the apoptosis figure. LR stand for the early apoptosis cells in lower right area of the apoptosis figure. LL stand for the viable cells in lower left area of the apoptosis figure. The value of apoptosis rate was expressed in the form of multiple increase.

RT-PCR. Trizol reagent was used for extracting total RNA. Intracellular RNA was then reverse transcribed into cDNA in accordance with the iScript cDNA synthesis kit. The expression of target genes were measured by SYBR Green real-time PCR Master Mix using the 2-ΔΔCt

Fig. 2. Effect of selenium on cell cycle distribution. Cell cycle distribution was detected by flow cytometry analysis (FACS) after pre-incubated with different concentration of selenium (40 μmol/L of SSE, 20 μmol/L of MSA, 100 μmol/L of MSC). One-way ANOVA was used for comparison between treatments for each stage. Data are expressed as mean±SD. Different characters in each set (G0/G1, S, G2/M) mean significant differences (p<0.05).

Fig. 3. Cell apoptosis induced by SSE (B), MSA (C) and MSC (D) (mean±SD, n=3). Cell apoptosis was measured by flow cytometry analysis (FACS) after treated with 40 μmol/L of SSE, 20 μmol/L of MSA or 100 μmol/L of MSC. One-way ANOVA was used for comparison between each treatment. Data are expressed as mean±SD. Different characters on the bar mean significant differences (p<0.05) versus control (A).
method with the internal control of GAPDH. Primers used in this study was displayed in Table 1.

**Western blot.** Proteins were extracted using RIPA buffer containing cocktail protease inhibitor. Total concentration of protein was measured by BCA assay. Protein samples (20 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene difluoride (PVDF) membranes. After blocked with 5% non-fat milk for 1 h, membranes were subsequently incubated with primary antibodies and HRP-conjugated secondary antibody. Signals were measured by the enhanced chemiluminescence (ECL) kit.

**Statistical analysis.** All experimental data were presented as mean±standard deviation. One-way ANOVA analyse was used to determine differences between groups with SPSS 19.0. p-values of less than 0.05 were considered statistically significant.

**RESULTS**

**Cell viability**

The regulatory effect of selenium compound on cell proliferation was detected by MTT assay. Results in Fig. 1 indicated that cell viability in groups treated by selenium was significantly lower than control group, after 24, 48 or 72 h incubation (p<0.05). While, with the prolongation of culture time, cell viability decreased gradually, and this tendency was also found in all groups of MSC with the increasing of dose. Also, the result value proved that MSC showed stronger inhibitory effects for Eca109 cell line than SSE and MSA (p<0.05).

**Cell cycle**

The cell cycle of Eca109 in this study was analyzed by flow cytometry analysis. Values showed in Fig. 2 suggested that cells were arrested at G0/G1 phase after incubated with MSA or MSC for 48 h, and cell population in S phase decreased in both MSA and MSC group when compared with the control (p<0.05). In addition, cell population in G2/M phase was reduced by MSC, but no alteration in MSA group. Finally, there was no sig-

![Fig. 4. Effect of selenium on genes expression of cyclinD1, CDK2, caspase-3, caspase-8, PTEN and FAL1 on Eca109 cells. The mRNA expression levels of Eca109 cells after incubated with different selenium (40 µmol/L of SSE, 20 µmol/L of MSA or 100 µmol/L of MSC) for 48 h were quantified by RT-PCR. Results are shown as relative expression ratio of the target gene in treatment group comparing with the control group and expressed in comparison to reference gene GAPDH. One-way ANOVA was used for comparison between treatments for each gene. Data are expressed as the mean±SD. Different characters in each set (cyclinD1, CDK2, caspase-3, caspase-8, PTEN and FAL1) indicate significant differences (p<0.05).](image)

![Fig. 5. Effect of selenium on protein expression of cyclinD1, CDK2, caspase-3, caspase-8 and PTEN. Cells were stimulated with 40 µmol/L of SSE, 20 µmol/L of MSA or 100 µmol/L of MSC for 48 h. The total cellular proteins were extracted and separated by SDS-PAGE. Specific antibodies raised against cyclinD1, CDK2, caspase-3, caspase-8 and PTEN were used in the Western blot assay with the GAPDH for normalization. One-way ANOVA was used for comparison between treatments for each protein. Data are expressed as mean±SD. Different characters in each set (cyclinD1, CDK2, caspase-3, caspase-8 and PTEN) indicate significant differences (p<0.05).](image)
nificantly change of cell cycle distribution in the group treated with SSE.

Cell apoptosis

Cell apoptosis was evaluated using flow cytometry assay. The results in Fig. 3 revealed that incubated with SSE, MSA or MSC for 48 h resulted in a significantly increase in cell apoptosis comparing with control \( (p<0.05) \). Noticeably, the apoptosis stimulated by MSC was much higher than that of SSE and MSA \( (p<0.05) \), and SSE showed moderate effect of promotion apoptosis.

Cell viability related mRNA and protein expression

The expressions of cell viability related mRNA (Fig. 4) and protein (Fig. 5), including FAL1, PTEN, cyclinD1, CDK2, caspase-3, and caspase-8 were detected by RT-PCR and Western blot respectively. All these selenium could reduce FAL1 expression, and increase PTEN level compared with control group \( (p<0.05) \). MSC and MSA showed stronger effect of decreasing cyclinD1 and CDK2 expression than SSE \( (p<0.05) \). On the other side, Caspase-3 and Caspase-8 were dramatically increased by SSE rather than MSC or MSA \( (p<0.05) \).

DISCUSSION

Increasing evidence has proved that, selenium is an effective anticancer agent, especially for the treatment of malignant tumors. Most cellular physiological functions of selenium are conducted by selenocysteine (Sec), which is the complex of selenium and selenoproteins. But the exact mechanism of selenium’s anti-cancer action is still confusing, partially because it contains both anti-neoplastic effectiveness and cytotoxic (14–16).

Generally, the effectiveness and toxicity of selenium compounds depend heavily on the concentration, chemical species and the redox potential (17). The most two common existence forms of selenium, inorganic selenium (such as SSE) and organic selenium (such as MSA, MSC) may exert bioactive or cytotoxic effects in vitro strictly depend on compound, concentration and model used. This may due to their different metabolic mechanism. For example, SSE could act as a precursor for the biosynthesis of other selenium compounds dependent on the availability of glutathione (GSH) and the presence of enzymes. While MSC has two different metabolic pathways. One is to enter the inorganic selenium metabolism pool, convert to H2Se as a precursor for the synthesis of various selenoproteins, at the same time there is another special metabolic pathway, MSC can transform into CH3SeCH3, and then methyl selenol is formed directly, which plays a strong biological effects in organisms.

In the present study, we found that SSE, MSA or MSC could consistently reduce the viability of EC cells with an obvious positive time-efficacy relationship. Notably, MSC show stronger inhibitory effect than SSE and MSA. However, this positive correlation was not completely consistent between concentration and cell activity. For example, there were no significantly changes of cell viability after 24 and 48 h of treatment with low and medium concentrations SSE; also no changes of the cell viability in groups treated with medium and high concentrations MSA for 72 h. Similar result was also been reported in previous study (18), which demonstrated that the viability of breast cancer cells unchanged or increased with the increasing concentration of MSC. This founding indicated that higher concentration of selenium doesn’t means stronger effect, using an appropriate concentration of selenium can maximize its anti-tumor effect for EC cells.

It reported that FAL1 has been associated with progression of various cancers, through regulating the proliferation, invasion, migration as well as apoptosis of tumor cells (19). For example, FAL1 promotes the proliferation of colon and gastric cancer cells via promoting Bcl-2 (20) and PTEN (21) respectively. Another study has reported that cell cycle was arrested in G0/G1 phase and cell apoptosis increased in FAL1 knockdown non-small cell lung cancer cells (22).

Here, we first found that FAL1 expression was reduced by all three types of selenium, particularly by MSC. Also, our findings indicated that PTEN, a target gene for FAL1 was stimulated in all groups. Low expression of PTEN occurs in various of malignant tumors (23). PTEN could inhibit the PI3K/AKT pathway which is an intracellular signaling pathway that takes crucial role in regulating cell cycle and apoptosis (24). Activated Akt promote cell proliferation through inhibiting p27 and p21 which were anti-proliferative proteins (25). Akt also restrain cell apoptosis by upregulating apoptosis-related genes Bcl-2 (26). Here, we determined the effect of selenium on cell cycle and apoptosis of EC. Consistently, cell cycle was arrested in G0/G1 phase, and cell population was increased in S phase after treated with MSC and MSA. But, there was no significant change of cell cycle distribution after cells incubated with SSE. Meanwhile, MSC showed stronger effect of promoting cell apoptosis than MSA and SSE.

Further, we analyzed the expressions of cell cycle and apoptosis related gene and protein to find out reasons for distinct inhibitory effect of different selenium. CDK2 and cyclinD1 were both reduced by MSC and MSA, but CDK2 was unchanged after cells treated with SSE, this probably because SSE interfere protein expression of cyclinE, which could form complex with CDK2, and subsequently affects CDK2 activity (27). Interestingly, caspase-3 and caspase-8 expression were dramatically promoted by SSE compared with MSC and MSA, this result may indicated that the anti-tumor effect of SSE probably due to its pro-apoptosis effect rather than anti-proliferation effect.

To sum up, we observed that selenium compound could reduce the proliferation and cell cycle of EC cells by regulating FAL1, and subsequently PTEN. Differently, MSC and MSA inhibit EC cell growth by inhibiting cycle-related proteins, while SSE was more effective in promoting apoptosis than MSA.
Authorship
Xiangsen Liang designed this study, Tao Liu and Yu Sun collected and analyzed the data, Tao Liu and Shengzhuang Yang wrote the manuscript, Yu Sun approved the final version of the manuscript. All authors read and approved the manuscript for publication.

Tao Liu and Yu Sun contributed equally to this study.

Disclosure of state of COI
The authors declare that they have no conflict of interests.

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