Se-Methylselenocysteine inhibits proliferation and migration of anaplastic thyroid carcinoma cells through the ROS-ERK1/2 signaling pathway

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Research Article
Abstract

Se-Methylselenocysteine (MSC) is a major organic selenium compound that possesses anticancer activity. However, the antitumor effect of MSC in anaplastic thyroid carcinoma (ATC) remains to be investigated. In this study, the two human ATC cell lines 8305 and BHT101 were used to examine the potential antitumor effect of MSC in ATC. Cell viability was measured using Cell Counting Kit-8, and the migration ability of the cells was evaluated by Transwell assays. The 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) was used to analyze reactive oxygen species production. Changes in extracellular regulated-protein kinases 1/2 (ERK1/2) and phosphorylation level of ERK1/2 proteins were analyzed by western blotting in 8505C and BHT101 cells. The results indicated that MSC dose-dependently inhibited proliferation and migration of ATC cells. MSC also decreased the phosphorylation level of ERK1/2 and increased intracellular reactive oxygen species (ROS) level in ATC cells. The inhibitory effect of MSC on ERK1/2 signaling was reversed by a ROS scavenger. In conclusion, MSC exerted its antitumor activity in ATC cells by inhibiting ERK1/2 signaling via a ROS-dependent pathway. Therefore, our results suggested that MSC had potential clinical value in the treatment of ATC.

Introduction

Selenium (Se) is one of the essential trace elements in the human body, and has a wide range of physiological activities. Different forms of selenium at different doses can exert antitumor or auxiliary antitumor effects through various mechanisms. The potential anticarcinogenic effect of selenium was first suggested in the late 1960s (Schrauzer et al. 1971). The antitumor properties of selenium have been demonstrated, and selenium supplements have been used in individual anticancer treatments (Tugarova et al. 2018). Recent studies show that selenium suppresses chicken hepatocellular carcinoma cell proliferation and metastasis (Hu et al. 2020). At high doses, selenium compounds inhibit neoplastic growth by producing reactive oxygen species (ROS) (Wallenberg et al. 2014). Generation of ROS is involved in selenium-mediated cytotoxic effects on cancer cells. Selenium exists in nature in two main forms, inorganic selenium and organic selenium. Several chemical forms of selenium including selenite, methylseleninic acid, and Se-methylselenocysteine (MSC) have anticancer potential as well as supranutritional levels (Li et al. 2008; Chen et al. 2013). MSC has distinct advantages as an organic compound, as demonstrated in several human cancer cell lines and animal models, where it shows few side effects and low systemic toxicity (Valdiglesias et al. 2010; Chen et al. 2013; Selvam et al. 2021). MSC increases ROS levels in HL-60 cells (Jung et al. 2001). The antitumor activity of MSC in HeLa cells is mediated by the inhibition of ERK signaling pathways (Sun et al. 2017), suggesting that a similar mechanism exists in thyroid cancer.

Thyroid carcinoma is one of the most common head and neck malignancies (Siegel et al. 2018), and anaplastic thyroid carcinoma (ATC) is a rapidly progressive and highly malignant disease with a dismal prognosis. Chemotherapy and radiotherapy are the main options for patients with ATC, although effective treatments are lacking. Although ATC accounts for only 1–3% of thyroid cancers, this form is associated with a mortality of 14–50% (Jemal et al. 2007). The average survival time of patients with ATC is 3–6
months (Xu et al. 2020; Leboulleux et al. 2021). Therefore, exploring new treatments for ATC may significantly improve the outcome of patients.

Extracellular signal-regulated protein kinase (ERK)1/2 belongs to the mitogen-activated protein kinase (MAPK) family, which has been proposed as the crucial regulators in the development of various cancers. ERK1 and ERK2 are two important members of the MAPK/ERK pathway that are 84% identical at the amino acid level and have the same acting substrate \textit{in vitro}. They are therefore referred to as ERK1/2. ERK1/2 regulates the growth, division and death of tumor cells and plays an important role in signal transduction pathways. Its overactivation promotes rapid proliferation and migration of tumor cells. (Zhao et al. 2018). Studies have shown that the ERK pathway is the main therapeutic target of thyroid cancer, and plays an important role in the occurrence and development of thyroid tumors (Xing et al. 2013). Thyroid cancer cells could be killed by inhibiting the MAPK/ERK pathways via a ROS-dependent mechanism (Su et al. 2019). In this study, we showed the antitumor effects of MSC in ATC cells using various \textit{in vitro} techniques. MSC inhibited the proliferation and migration of ATC cells by inactivating the ERK1/2 signaling pathway via a ROS-dependent mechanism.

**Materials And Methods**

**Reagents and antibodies**

The selenium compound MSC was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from HyClone (USA). Cell Counting Kit-8 was purchased from Gibco (USA), and DCFH-DA was obtained from Beyotime (Shanghai, China). Antibodies against ERK1/2 and p-ERK1/2 were obtained from Cell Signaling Technology (USA).

**Cell cultures**

The human ATC cell lines 8305C and BHT101 were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China). The 8305C and BHT101 cells were maintained in DMEM (Procell, China) supplemented with 10% FBS (Gibco, USA) and 100 U/mL penicillin-streptomycin (Solarbio, Beijing, China). All cells were incubated at 37°C and 5% CO$_2$ in a cell incubator.

**Cell viability assay**

MSC was diluted with DMEM to concentrations of 25, 50, 100, 150, 200, and 300 µM. After incubation with MSC for 24 h, BHT101 and 8305C cells were treated with 100 µL DMEM and 10 µL Cell Counting Kit-8. The plates were covered with foil to protect them from light and incubated at 37°C for 1 h. The optical density (OD) was measured by a SynergyH1 reader (BioTek, USA) at 450 nm. Cell viability was calculated according to the following formula: cell viability (%) = (average OD value of the experimental group - average OD value of the Blank group)/(average OD value of the control group - average OD value of the blank group) \times 100%.

**Migration assay**
The treated cells were placed in the upper chamber of a 24-well Transwell cell culture chamber plate (Corning Incorporated, USA) at a concentration of $5 \times 10^4$ cells per well in 100 µL DMEM serum-free medium. The lower chamber contained 800 µL of DMEM supplemented with 20% FBS. Cells were treated or not with MSC. After 24 h, the plate was washed with PBS, and 4% paraformaldehyde was added to fix the samples at room temperature. After 30 min, the chamber was removed, washed once with PBS, and then 0.5 mL 0.1% Crystal Violet dye was added for staining at room temperature. Then, 10 min later, PBS was added for rinsing, then chambers were wiped gently with a cotton swab to remove the upper layer of unpenetrated cells, and air dried. Images of cell penetration were captured using an inverted microscope (Nikon, Tokyo, Japan).

**ROS level measurement**

Treated cells were washed twice with PBS. A solution of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime, Shanghai, China) was diluted with serum-free medium at a ratio of 1:1,000, then 1 mL of diluted DCFH-DA was added to each well, and the well plate was wrapped in tin foil and incubated in the incubator without light. After 20 min, the cell culture plate was removed, and serum-free culture medium was added to wash the cells, then the procedure was repeated three times. Finally, 1 mL of serum-free medium was added to each well, and the fluorescence intensity was observed and photographed using a fluorescence microscope (Nikon) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. In some experiments, 5 mM N-acetylcysteine (NAC) was added to remove cellular ROS

**Western blot analysis**

MSC was used to treat ATC cells for 24 h. In some experiments, 5 mM NAC was used to eliminate intracellular ROS production. Total cell protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Proteins were separated by SDS-PAGE (Bio-Rad, Hercules, CA, USA) and then transferred to PVDF membranes (Millipore, Burlington, MA, USA) with primary antibody diluents of ERK1/2, and p-ERK1/2 (both 1:500 dilutions) and incubated overnight at 4°C. After primary antibody incubation, PVDF membranes were washed with TBS-T buffer and incubated with horseradish peroxidase-conjugated secondary antibody (Signalway Antibody, Greenbelt, MD, USA) at 37°C. After 1 h, PVDF membranes were washed three times with TBS-T buffer cartridges. Subsequently, the proteins were confirmed by visualization using a LI-COR system (Odyssey, Lincoln, NE, USA). The intensity of the bands of interest were analyzed with ImageJ software, version 1.8.0 (National Institutes of Health, Bethesda, MD, USA). An anti-β-tubulin antibody (Abmart, Shanghai, China) was used as an internal control.

**Statistical analysis**

The experimental data were analyzed using SPSS statistical software for Windows, version 23.0 (SPSS, Chicago, IL, USA), and the results were expressed as the mean ± SD. Graphs were generated using Prism 7.0 (GraphPad, San Diego, CA, USA) and ImageJ, version 1.8.0. Differences between groups were analyzed using one-way analysis of variance. $P < 0.05$ and $P < 0.01$ were considered statistically significant.
Results

Cell line characterization

The 8305C line is comprised of epithelioid cells with a long spindle shape (Fig. 1A), whereas BHT101 cells are round in shape and have hyper-chromatic nuclei (Fig. 1B).

MSC inhibits the viability of ATC cells.

MSC treatment for 24 h significantly inhibited the viability of 8505C and BHT101 ATC cells (*P < 0.05, **P < 0.01 vs. control) (Fig. 2).

MSC suppresses the migration of ATC cells.

MSC at concentrations of 0, 100, 150, and 200 µM inhibited the migration of 8305C and BHT101 cells in a dose-dependent manner (Fig. 3). The cells in both chambers were counted. MSC, particularly at 200 µM, suppressed the motility of ATC cells.

MSC inhibits the phosphorylation of ERK1/2 in ATC cells.

It is thought that the ERK1/2 signaling pathway plays an important role in the development of thyroid cancer. We therefore examined the effect of MSC on the activity of this pathway in ATC cells. As shown in Fig. 4A and B, MSC treatment markedly inhibited ERK1/2 signaling in ATC cells, as indicated by decreased phosphorylation of ERK1/2 in a dose-dependent manner.

MSC inhibits ERK1/2 pathways in ATC cells by inducing cellular ROS production.

Considering that MSC treatment kills cancer cells by inducing the production of endogenous ROS, we measured cellular ROS levels in thyroid cancer cells treated with MSC for 2–4 h using dichloro-fluorescein (DCF) staining. MSC treatment significantly increased cellular ROS levels in 8305C and BHT101 cells (Fig. 5A–D). The inhibitory effect of MSC on ERK 1/2 signaling was reversed by NAC treatment (Fig. 5E–H). These data indicated that MSC blocked the ERK1/2 signaling pathway through a ROS-dependent mechanism.

Discussion

ATC is one of the most aggressive solid tumors in humans and is associated with a poor prognosis. The clinical course of ATC is characterized by rapid tumor progression, local invasion and/or distant metastases, and rapidly fatal clinical outcomes. Most patients die from distant metastatic disease (Onoda et al. 2020; Bible et al. 2021; Dierks et al. 2021). Currently, there are no effective treatments to cure or to prolong the survival of patients with ATC. Selenium exhibits toxicity against cancer cells. MSC, a form of organic selenium, has shown anticancer properties in preclinical experiments and clinical studies, although the underlying mechanism remains unclear.
In this study, we investigated the role of MSC in regulating the proliferation and migration of ATC cells. The results indicated that MSC inhibited the proliferation and migration of ATC cells and decreased the activity of ERK1/2 pathways by inducing cellular ROS production, indicating that MSC had antitumor activity against ATC.

Our results showed that MSC decreased the proliferation and migration of 8305C and BHT101 cells. The organ with the highest selenium content in the body is thyroid tissue. Selenium, as the active center of selenoproteins, plays an important role in thyroid metabolism and other physiological processes (Duntas et al. 2015; Gorini et al. 2021). Selenium is indispensable in the synthesis, secretion, and metabolism of thyroid hormone. A meta-analysis (Shen et al. 2015) that included four studies on the relationship between serum selenium levels and thyroid cancer showed that decreased serum selenium levels were associated with thyroid cancer. Serum selenium levels were significantly lower in thyroid cancer patients than in controls (Baltaci et al. 2017). Other studies have come to the same conclusion (Glattre et al. 2012). MSC inhibits the proliferation of tumor cells by affecting the G1, G2, and S phases of the cell cycle (Zeng et al. 2009). MSC can inhibit the migration of mouse breast tumor cells TM6 (Unni et al. 2004). In a nude mouse human colon cancer model, MSC decreased the blood volume and microvessel density of transplanted tumors (Bhattacharya et al. 2011). The results of the present study are consistent with these previously published results.

ERK-related intracellular signaling pathways are considered classical MAPK pathways that are responsible for extracellular stimulation. Signal transduction from the cell membrane to the nucleus through an enzymatic cascade of a series of kinases is an important link. Activation of the ERK pathway is associated with the development of numerous tumors (Nickols et al. 2019). The ERK1/2 signaling pathway is involved in cell proliferation, differentiation and other important physiological processes (Oba et al. 2012; Dufey et al. 2021). Generally, promoting tumor cell apoptosis or inhibiting cell proliferation requires downregulation of the ERK signaling pathway. Inhibition of MAPK/ERK signaling in esophageal squamous cell carcinoma and ovarian cancer can weaken tumor invasion and metastasis (Chang et al. 2012; Gao et al. 2019). ERK1/2 inhibition results in enhanced inhibition of thyroid tumor growth in vivo (Hicks et al. 2021). Given the central role of ERK1/2 signaling pathways in thyroid tumorigenesis and malignant progression, we investigated the effect of MSC on this pathway to elucidate its antitumor mechanisms. The results showed that MSC treatment markedly inhibited ERK1/2 signaling in ATC cells, as demonstrated by decreased phosphorylation of ERK1/2 in a dose-dependent manner. The inhibitory effect of MSC on the ERK1/2 signaling pathway could be reversed by NAC treatment.

During the rapid proliferation of cancer cells, metabolic stress increases, and the level of basal ROS in cells is often higher than that in normal cells. Therefore, tumor cells are more sensitive to ROS accumulation, and the induction of ROS accumulation can serve as an important mechanism for killing of tumor cells by drugs. Medium-to-high doses of selenium compounds act as pro-oxidants to interfere with intracellular redox balance, promoting the production of ROS such as superoxide anion and $H_2O_2$ (Misra et al. 2015; Jia Ma et al. 2021). ROS are important regulatory molecules related to the survival and death of tumor cells. Moderate ROS production may be beneficial to cell survival, but excessive ROS
interfere with cell signaling pathways, thereby causing oxidative damage to cells. (Moloney et al. 2018). Intracellular ROS can oxidize the cysteine residue of ERK1/2, thereby inactivating it and causing loss of function (Luanpitpong et al. 2012). This study showed that MSC significantly increased ROS production in ATC cell lines 8305C and BHT01. MSC-induced ROS decreased ERK1/2 phosphorylation levels, cell viability, and migration ability, and these effects were verified by NAC treatment.

Conclusion

In summary, we performed a series of in vitro studies to demonstrate the ROS-dependent killing of ATC cells by MSC. MSC induced ROS-dependent inhibition of ERK1/2 signaling in ATC cells, to provide a theoretical basis for the clinical treatment of MSC in ATC.

Abbreviations

MSC  Se-(Methyl)selenocysteine
ATC  anaplastic thyroid carcinoma
CCK-8  Cell Counting Kit-8
DCFH-DA  2,7-dichlorodihydrofluorescein diacetate
ERK1/2  extracellular regulated-protein kinases 1/2
ROS  reactive oxygen species

Declarations

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Author contributions

Bixiu Ban, Zuojie Luo, Yan Ma conceived and designed the study. Material preparation, data collection and analysis were performed by Bixiu Ban, Haiyan Yang, Zhenxing Huang, Yaqi Kuang, Yuping Liu, Xizhen Wu, Decheng Lu, Li Li, Jing Xian, Yingfen Qin, Xinghuan Liang. The first draft of the manuscript was written by Bixiu Ban, Zuojie Luo, Yan Ma carried out writing review and editing. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: All authors declare that they have no conflicts of interest.

References


Figures

Figure 1
Cell line identification of ATCC cells. (A, B) The cellular morphology of 8305C and BHT101 cells (10×, scale bar is 400 μm).

Figure 2
The effect of Se-methylselenocysteine (MSC) on the proliferation of ATC cells. The 8305C and BHT101 cells were cultured with MSC at concentrations of 0, 25, 50, 100, 150, 200, and 300 μM for 24 h. Each experiment was repeated at least three times. *P < 0.05; **P < 0.01 vs. control cells.
**Figure 3**

MSC suppresses the migration of ATC cells. The migration (A and B) of ATC cells after treatment with or without MSC (×10, scale bar = 400 μm). (C and D) The number of migrated cells was quantified, and the data are presented as the mean ± SD. **P < 0.01 vs. the control group.**
Figure 4

Protein expression of p-ERK1/2 was reduced in 8305 (A and C) and BHT101 (B and D) cells after MSC treatment. The data are presented as the mean ± SD. *P < 0.05, **P < 0.01 vs. the control group (MSC 0 µM group).
Figure 5

MSC ROS-dependently inhibits the activity of ERK1/2 signaling. MSC increased intracellular ROS level. A and B: ROS was measured using the fluorescent probe DCFH-DA and imaged by fluorescence microscopy (×20; scale bar=200 μm). C and D: The average fluorescence intensity was calculated by ImageJ software and reflects the intracellular ROS level. E-H: Expression of ERK1/2 and p-ERK1/2 in 8305C and BHT101
cells treated with MSC in the presence or absence of NAC. Data are presented as the mean ± SD. **P < 0.01 vs. the control group (MSC 0 µM group), ##P < 0.01 vs. the MSC group.