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ORIGINAL ARTICLE

Differential apoptotic response of human cancer cells to organoselenium compounds

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Abstract
Purpose Selenium (Se) compounds are well known to inhibit cell proliferation and induce cell death in human cancer cells. Respective chemical forms of Se are intracellularly metabolized via complicated pathways, which target distinct molecules and exhibit varying degrees of anti-carcinogenicity in different cancer types; however, the precise mechanisms by which Se activates apoptosis remain poorly understood.
Methods The effects of Se compounds, Se-methylselenocysteine (MSC), selenomethionine (SeMet), and selenite on cell proliferation, apoptosis and its pathway in established human carcinoma cell lines (HSC-3, -4, A549, and MCF-7) were investigated. Cancer cells were treated with each Se compound during different periods. Cell apoptosis, caspase activity and ER stress markers were analyzed by flow cytometric or immunoblotting analysis, respectively.
Results We examined four cell lines for their sensitivity to MSC and SeMet in comparison with selenite. SeMet increased apoptotic cells in p53-positive A549 cells, whereas MSC increased apoptotic cells in p53-mutated HSC-3 cells. High activities of caspase-3, -8 and -9 were observed during apoptosis, and a pan-caspase inhibitor, z-VAD-fmk, rescued the cell viability of HSC-3 cells exposed to MSC. In addition, the occurrence of endoplasmic reticulum (ER) stress was suggested by the observation that levels of phosphorylated eIF2α and caspase-12 activity are increased in Se-treated cells. Selenite and MSC were accompanied with the concurrent reduction of phosphorylated Akt levels, and the inhibitory effects of these Se compounds on vascular endothelial growth factor expression were observed with identical patterns.
Conclusion The present findings demonstrate that Se-induced apoptosis in carcinoma cells is basically a caspase-dependent process involving complicated mechanisms. Activation of both the intrinsic apoptotic pathway and ER stress pathway plays a major and concurrent role, while p53 activation seems to have only a functional role in SeMet.

Keywords Organoselenium compounds · Apoptosis · Endoplasmic reticulum stress · Caspase-12 · p53 · Angiogenesis

Introduction

Cancer chemoprevention has been defined as the use of dietary and pharmacological interventions with specific natural or synthetic agents designed to prevent, suppress, or reverse the process of carcinogenesis before the development of malignancy [1]. Natural dietary agents have drawn a great deal of attention due to their demonstrated ability to suppress various phases of carcinogenesis. Selenium (Se) is a ubiquitous metalloid with properties similar to those of sulfur, which have benefits in preventing several types of cancer, including lung, colorectal, head and neck and prostate cancers, but not breast and skin cancers in humans

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[2]. Recently, besides cancer chemopreventive activity, there is emerging evidence indicating the potential of Se compounds in cancer chemotherapy [3]. Among the proposed mechanisms, cell growth inhibition and apoptosis are postulated to be critical in cancer chemotherapy; however, the precise mechanisms by which Se activates apoptosis remain poorly understood. Moreover, it is also important to identify which specific molecular targets are used by Se to inhibit carcinogenesis.

Chemical derivatives of Se include inorganic compounds, such as selenite and selenate, and organic compounds, such as selenomethionine (SeMet) and selenocysteine. Previous studies have shown that the dose, chemical form and metabolic activity are determinants of anti-cancer activities of Se compounds [4]. Sodium selenite, a common dietary form of Se, can effectively induce a number of cancer cell lines to undergo apoptosis, raising the potential for its clinical application; however, apoptosis has been shown to occur as a result of inorganic Se toxicity, causing DNA strand breaks and leading to chromosomal damage in carcinoma cell lines and human lymphocytes, respectively [5]. Organic Se compounds have fewer side effects and lack the genotoxic action of inorganic Se compounds, such as selenite, which directly enter the methylated pool through methylselenol, the presumed active metabolite responsible for the anti-cancer activity of Se [6]. SeMet is one of the most important dietary forms of Se in humans and, therefore, it is commonly used in cancer prevention studies [7]. Although excessive intake of SeMet is toxic, its upper safety limit is not well defined [8]. Se-methylselenocysteine (MSC) has distinct advantages as an organic compound, including its superior in vivo efficacy, non-existent toxicity, low body accumulation and simple formulation [9]. Practically no data exist to explain why certain forms of Se are more active than others in the inhibition of cancer cell growth.

It is well known that apoptotic cascades can be triggered by either extrinsic receptor-mediated, intrinsic mitochondria-mediated or endoplasmic reticulum (ER) stress-mediated signaling pathways. Se induces cellular apoptosis, presumably by acting on the functions of many intracellular proteins important for these apoptotic cascades. Se compounds induce caspase-mediated apoptosis in cancer cells, which is associated with decreased phosphorylation of Akt/PKB and ERK1/2 [10]. The PI3K/Akt pathway has been shown to inhibit apoptosis in most cell types and promote angiogenesis [11]. Anti-angiogenic activity may be a novel mechanism contributing to anti-cancer activity of Se. The tumor suppressor p53 can induce apoptosis by activating the expression of apoptosis-related genes in the nucleus, or by directly permeabilizing mitochondria in the cytoplasm [12]. Certainly, several studies have demonstrated that p53 and its phosphorylation were involved in the chemopreventive action of selenite, SeMet and methylseleninic acid [7]. In general, ER stress rapidly increases phosphorylation of the translation initiation factor eIF2α through activation of the ER kinase PERK. Cancer cells with compromised PERK and eIF2α signaling are substantially more sensitive to ER-induced cell death than wild-type cells [13]. Methylseleninic acid caused global thiol/disulfide redox modification of numerous proteins distributed in ER [14]. These changes are expected to lead to protein unfolding or misfolding, especially for newly synthesized proteins. Thus, it is highly plausible that certain Se produces ER stress; however, the precise mechanisms by which Se induces apoptosis through ER stress remain poorly understood.

In this study, we report experiments comparing the apoptotic mechanisms of SeMet and MSC with selenite against human oral squamous cell carcinoma cells, breast cancer cells, and lung carcinoma cells. Furthermore, we tested the hypothesis that Se induction of apoptosis is linked to the p53 pathway and ER stress. We observed that Se-induced apoptosis occurred in a caspase-dependent manner, accompanied with ER and mitochondria stress. These pleiotrophic effects of Se make it unique as a chemotherapeutic modulator.

Materials and methods

Reagents

Selenite, SeMet and MSC were purchased from Sigma (St. Louis, MO, USA). ER stressor thapsigargin (TG), ER stress inhibitor salubrinal (Sal), p53 inhibitor pifithrin-α (PFT), phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (LY), and calpain inhibitor SJA6017 (SJA) were purchased from Calbiochem (La Jolla, CA, USA). Hypoxia mimic reagent cobalt chloride ($CoCl_2$) was obtained from Sigma. All other chemicals used in this study were commercially available. All inhibitors used in this study were pretreated for 1 h, followed by each indicated treatment without washing.

Cell lines and cell culture

HSC-3 cells, HSC-4 cells (mutant-type p53, human oral squamous cell carcinoma), A549 cells (wild-type p53, human non-small-cell lung adenocarcinoma), and MCF-7 cells (wild-type p53, human breast cancer), were obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Japan). Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA).

Cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Cytotoxicity assays

Cell viability was evaluated by the trypan blue exclusion assay, and then the cytotoxic effects of Se derivatives were determined using an MTT (WST-8) colorimetric assay kit (Dojindo, Kumamoto, Japan) as described previously [15].

TUNEL assay

Apoptotic cells were assayed by the TUNEL method using the Mebstain apoptosis kit direct (MBL, Nagoya, Japan) for flow cytometric analysis (FACSCalibur; Becton–Dickinson, San Jose, CA, USA).

Measurement of intracellular caspase activity

Intracellular caspase-3, -8, -9 and -12 activities were measured using fam-DEVD-fmk, fam-LETD-fmk, fam-LEHD-fmk (Cell Technology, Mountain View, CA, USA) and FITC-ATAD-fmk (Abcam, Cambridge, MA, USA), respectively. Labeled cells were analyzed by flow cytometry. Inhibitor of pan-caspase (z-VAD-fmk) was purchased from MBL. A DMSO control was also included as a control for the given concentration of the inhibitor.

Western blotting

Cancer cells were treated with each Se compound at doses indicated for the times indicated. After treatment, whole or nuclear proteins were analyzed by Western blotting. Immunodetection was accompanied using HRP-conjugated secondary antibodies and an enhanced chemiluminescence detection method. The primary antibodies against BiP/GRP78 (Ana Spec, San Jose, CA, USA), phospho-eIF2α (Ser^{51}), total-eIF2α, p53 (Cell Signaling, Danvers, MA, USA), β-actin (BioVision, Mountain View, CA, USA), CHOP (Affinity BioReagents Inc, Golden, CO, USA), and HIF-1α (BD Biosciences, San Jose, CA, USA), were used. HRP-conjugated secondary antibodies, sheep anti-mouse IgG (GE Healthcare, Piscataway, NJ, USA) and goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used.

Measurement of Akt phosphorylation

The amounts of phosphorylated Akt relative to the total amounts of Akt in cells were measured with an ELISA kit (Active Motif, Carlsbad, CA, USA) as described previously [15]. Results are expressed as absorbance at 450 nm.

HIF-1α assay

To analyze HIF-1α/HRE (hypoxia response element) interaction, an ELISA-based assay was carried out according to the manufacturer's instructions (Active Motif) as described previously [15]. Results are expressed as absorbance at 450 nm.

Measurement of VEGF-A secretion

Cells (2×10^4 cells/well) were seeded in 12-well flat bottomed culture plates, incubated in medium with 10% FBS for 24 h, and then washed and treated with Se compounds in 1% FBS media for 4 days (serum deprivation). The amounts of vascular endothelial growth factor-A (VEGF-A) were determined by ELISA kits (R&D Systems, Minneapolis, MN, USA). Results are expressed as the amount of VEGF-A/well.

Statistical analysis

Data are given as the mean values ± SE. Multiple comparisons were made by Scheffe's test. *P* values less than 0.05 were regarded as significant.

Results

Cytotoxicity of Se compounds in carcinoma cell lines

We first examined four established human carcinoma cell lines for their sensitivity to MSC or SeMet in comparison with selenite using MTT cytotoxicity assays. Cells were treated with 1–100 μM selenite for 48 h or with 10–1,000 μM MSC or SeMet for 48 h. All four cell lines were highly sensitive to selenite, which induced dose- and time-dependent growth inhibition (Figs. 1a, 2a). MSC was effective against three of the cell lines, HSC-3, HSC-4 and MCF-7, but showed poor or slow potency against the A549 line (Figs. 1b, 2b). Because significant inhibition occurred in HSC-3 cells treated with 5 μM (selenite) or 50 μM (MSC) and higher doses of the respective Se, these doses were used in each of the subsequent experiments; however, the inhibitory effect of 48 h treatment is less prominent for SeMet than other Se compounds (Fig. 1c). When A549 cells were examined for their sensitivity to SeMet, SeMet (50 μM) was able to significantly suppress the growth of A549 cells with prolonged incubation (4 or 5 days) (Fig. 2b).

Induction of apoptosis by Se compounds in HSC-3 cells

To determine whether Se-induced inhibitory effects in HSC-3 cells involved apoptosis, we analyzed the induction

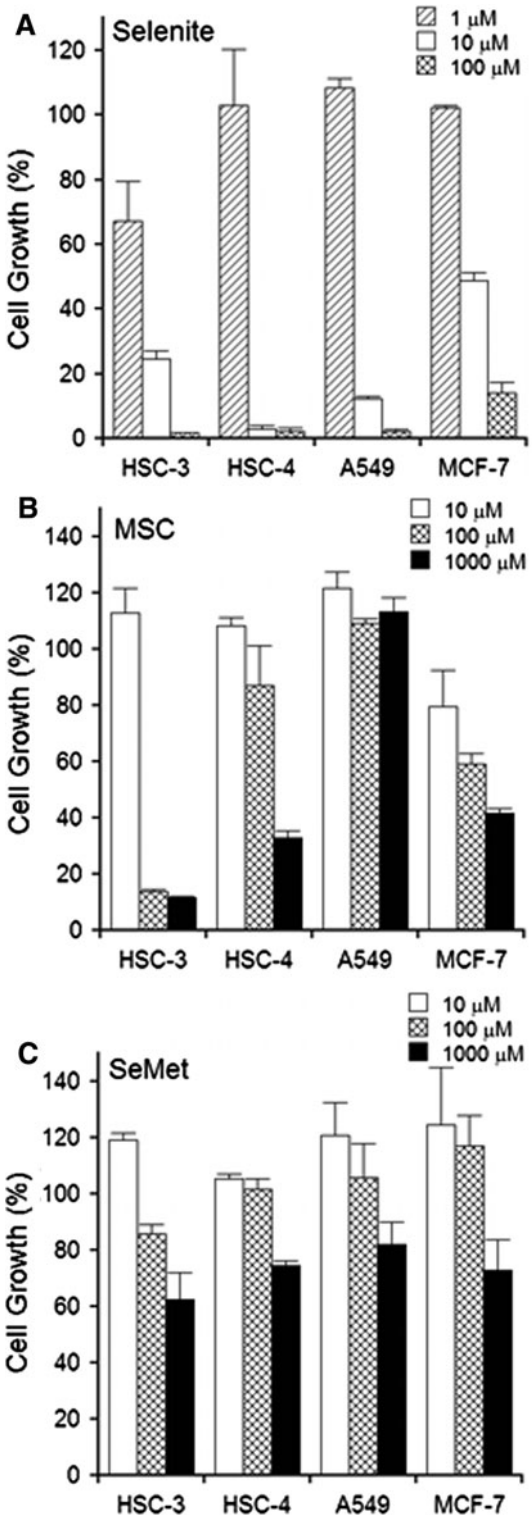

Fig. 1 Cytotoxicity of Se compounds in carcinoma cell lines. Respective cancer cell lines (2×10^4 cells/well) were treated with increasing concentrations of selenite (**a**), MSC (**b**), or SeMet (**c**) for 48 h, followed by the MTT assay. Results in triplicate represent the percentages (mean values ± SE) of cell growth to the untreated control

of nucleosome fragmentation by TUNEL staining. As shown in Fig. 3a, selenite treatment for 2 and 5 days induced significant apoptosis at a dose of 5 μM in HSC-3 cells. With regard to apoptosis, MSC (50 μM) treatment for 5 days induced apoptosis moderately, but SeMet did not lead to significant apoptosis at 50 μM relative to the other compounds. In addition, we investigated the possible pathways responsible for the apoptotic effects of selenite and MSC. To establish the key caspases involved in Se-induced apoptosis, we examined the activation of caspase-3, -8, and -9 in Se-treated HSC-3 cells. Each treatment activated all caspases tested, and cell killing by MSC was partially blocked by a pan-caspase inhibitor (z-VAD-fmk) (Fig. 4a, b), indicating that cell death by these Se compounds is caused by a caspase-dependent apoptotic pathway. The activation of caspase-9 indicated that mitochondrial pathways might be involved in both Se-induced cell apoptosis in HSC-3 cells, and caspase-8 may participate in the mitochondrial amplification loop [16].

p53-dependent apoptosis by SeMet in A549 cells

It was evaluated whether there is any differential apoptotic action for SeMet with respect to the p53 status. We found that SeMet or selenite induces cell apoptosis of p53-positive A549 cells (Fig. 3b, d). At 5 days, SeMet (50 μM) treatment caused the percentage of apoptotic cells to increase from 1.4 to 51.5% of the cellular population, whereas MSC did not induce significant apoptosis. In p53-negative HSC-3 cells, no significant changes in cell apoptosis were observed after 5 days of treatment with SeMet (Fig. 3a). To determine the possible role of p53 in apoptosis by SeMet, we examined the effect of pifithrin-α (PFT) on SeMet-induced apoptosis for 5 days in A549 cells. PFT (50 μM) clearly reduced apoptosis by SeMet in A549 cells (Fig. 3c). Thus, there is a difference in apoptosis induction between p53 blocked and unblocked cells in response to SeMet. Next, we showed that the relative activities of caspase-3, -8, and -9 in A549 cells were significantly increased by SeMet treatment (Fig. 4a). Despite the response to different forms of Se, cells treated with each Se displayed similar caspase activities typical of a robust apoptotic response.

Effects of Se compounds on ER stress pathway

In light of evidence that Se compounds induce ER stress responses in human cancer cell lines [14], we investigated Se-induced ER stress and its role in the observed apoptosis. When treated with SeMet at 10 μM for 4 days, a hallmark of ER stress-mediated apoptosis, caspase-12, was found to

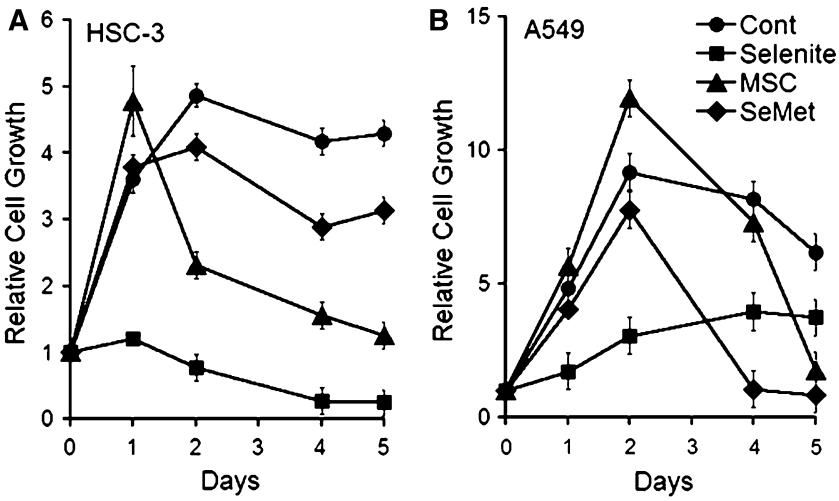

Fig. 2 Time kinetics of Se effects on cell growth. HSC-3 (**a**) or A549 (**b**) cells were seeded and incubated for 24 h (0 day). Cells were then treated with selenite (5 μM), MSC (50 μM) and SeMet (50 μM) for 5 days, followed by the MTT assay at each time point. Results are expressed as relative cell growth to control at 0 day

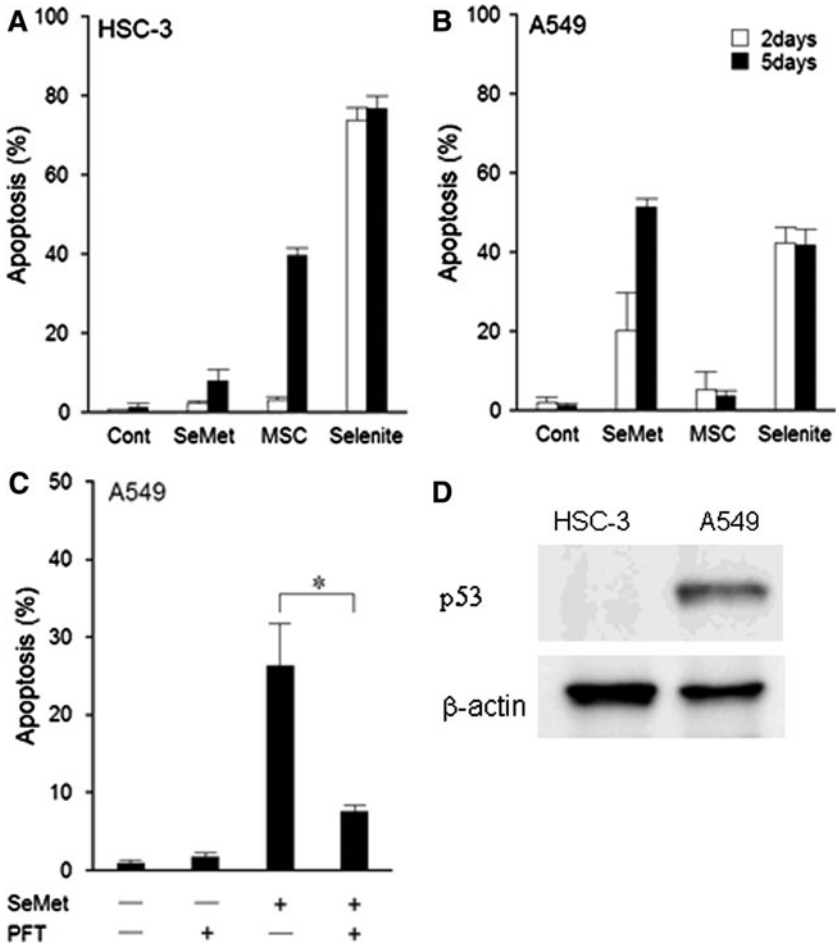

Fig. 3 Induction of apoptosis by Se compounds. HSC-3 (**a**) or A549 cells (**b**) were treated with selenite (5 μM), MSC (50 μM) and SeMet (50 μM) for 2 or 5 days, followed by the TUNEL assay. **c** A549 cells pretreated for 1 h with pifithrin-α (PFT, 50 μM) were treated with SeMet (10 μM) without washing for 5 days, and then subjected to the TUNEL assay. Fluorescence-positive cells, including the apoptotic subpopulation, were quantified. Results are expressed as the mean values ± SE of triplicate experiments. $^*P < 0.05$ versus SeMet alone. **d** Total cell lysates were prepared for p53 expression, and equal amounts of extracts were loaded for Western blotting. β-actin was used as a loading control

be activated in A549 cells (Fig. 5a). Caspase-12 was also activated in either selenite- or MSC-treated HSC-3 cells. In addition, A549 cells were pretreated with the calpain inhibitor SJA6017, followed by the TUNEL assay. SJA6017 significantly increased cell survival compared with SeMet alone after 5 days (Fig. 5b).

When compared with eIF2α phosphorylation of HSC-3 cells as an adaptive response to ER stress, only selenite consistently induced p-eIF2α at 6 h, while significant signals of p-eIF2α were not observed in MSC treatment (Fig. 6). Surprisingly, TG did not induce the phosphorylation of eIF2α under this condition. Furthermore, we tried

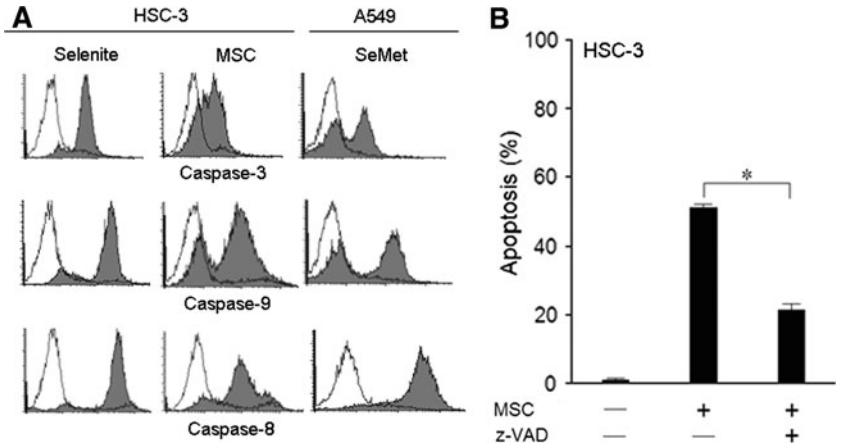

Fig. 4 Increased activity of caspases induced by Se compounds. **a** HSC-3 cells were treated with selenite (5 μM) for 24 h or MSC (100 μM) for 48 h. A549 cells were treated with SeMet (10 μM) for 4 days. Intracellular caspase activities (caspase-3,-8 and -9) were then analyzed by flow cytometry. Results show representative flow cytometric histograms depicting caspase active cells in untreated (unshaded) or treated cells (shaded). **b** HSC-3 cells pretreated for 1 h with z-VAD-fmk (20 μM) or DMSO alone were treated with MSC (50 μM) for 5 days, and then subjected to the TUNEL assay. Results are expressed as the mean values ± SE of triplicate experiments. $*P < 0.05$ versus MSC alone

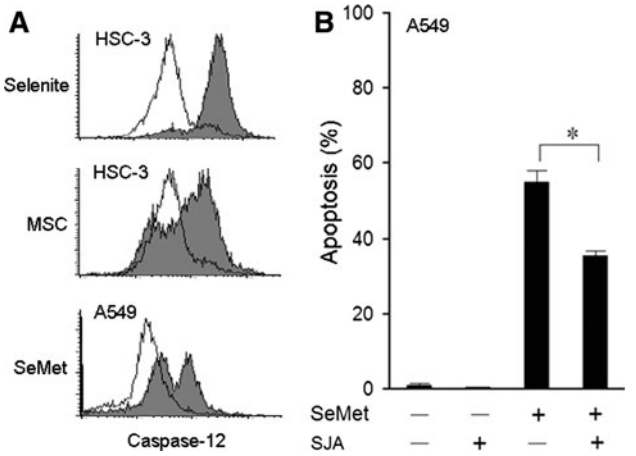

Fig. 5 Involvement of ER stress in Se-induced apoptosis. **a** HSC-3 cells were treated with selenite (5 μM) for 24 h or MSC (100 μM) for 48 h. A549 cells were treated with SeMet (10 μM) for 4 days. Intracellular caspase-12 activity was analyzed by flow cytometry. Results show representative flow cytometric histograms. **b** A549 cells pretreated for 1 h with calpain inhibitor SJA6017 (SJA, 50 μM) were treated with SeMet (50 μM) for 5 days, and then subjected to the TUNEL assay. Results are expressed as the mean values ± SE of triplicate experiments. $*P < 0.05$ versus SeMet alone

to detect phosphorylation of eIF2α, and detected its significant signals in A549 cells treated with SeMet for 24 h (Supplementary Fig. S1). We also monitored the expression of the pro-survival chaperone BiP/GRP78, and the pro-apoptotic transcription factor CHOP. Se treatments did not cause a major change in BiP/GRP78 and CHOP protein levels. We then investigated whether the inhibitor of ER stress could rescue Se-treated cells from apoptosis. Salubrinal (Sal), an established ER stress inhibitor, can inhibit eIF2α dephosphorylation and protect cells against ER stress-mediated apoptosis [17]. As shown in Fig. 7b, after pretreatment with 50 μM Sal, the cell apoptosis of SeMet-treated A549 cells decreased from 55.0 to 9.7%. In contrast, treatment of HSC-3 cells with Sal/MSC augmented the apoptotic effect of MSC (Fig. 7a). Toxicity of Sal itself was minimal for each cell at the concentrations used in all experiments. These observations suggested that alteration of Se-induced apoptosis by Sal is due to the balance of predominant pro-apoptotoic or pro-survival mediators regulated by ER stress.

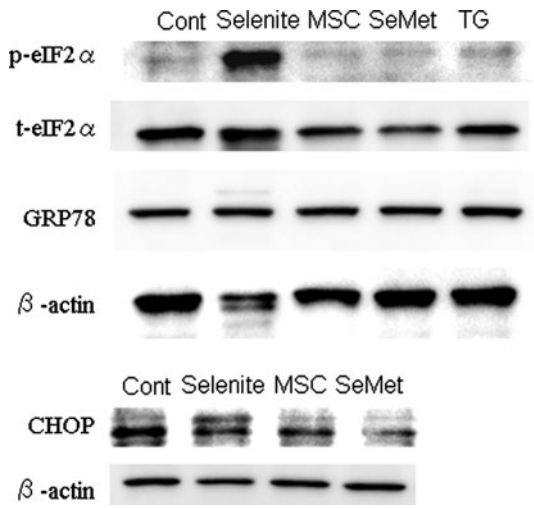

Fig. 6 Increased expression of phospho-eIF2α in cells treated with Se compounds. HSC-3 cells were treated with selenite (10 μM), MSC (100 μM), SeMet (100 μM) and thapsigargin (TG, 2 μM) for 6 h. Total cell lysates or nuclear lysates were prepared for expression of ER stress-related proteins, and equal amounts of extracts were loaded for Western blotting with the indicated antibodies

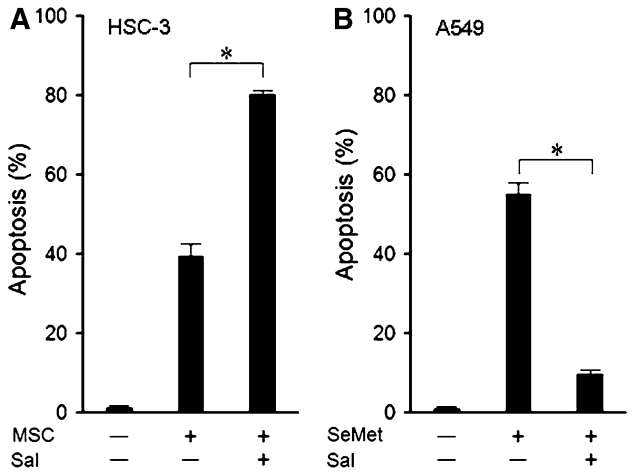

Fig. 7 Opposite effects of salubrinal on apoptosis induced by MSC or SeMet. HSC-3 (**a**) or A549 (**b**) cells pretreated for 1 h with salubrinal (Sal, 50 μM) were treated with MSC (50 μM) or SeMet (50 μM) for 5 days, and then subjected to the TUNEL assay. Results are expressed as the mean values ± SE of triplicate experiments. $^{*}P < 0.05$ versus MSC or SeMet alone

Effects of Se compounds on Akt signaling pathways

The Akt signaling pathway, a well-known pro-survival pathway, has recently been shown to counteract ER stress-induced cell death [18]; therefore, we compared the phosphorylation status of Akt to determine whether SeMet or MSC exposure produced biochemical action profiles similar to those of selenite. Akt (Ser^{473}) phosphorylation was detected in untreated HSC-3 cells. Selenite or MSC, like the PI3K inhibitor LY294002, decreased the phosphorylation level of Akt in HSC-3 cells, and SeMet did not in unresponsive cells (Fig. 8a). These inhibitory effects appeared to be proportional to the extent of apoptosis induced by each Se (Fig. 3a).

Serum deprivation activates the PI3K/Akt pathway, which, in turn, increases HIF-1α protein. Finally, Akt signaling mediates the secretion of pro-angiogenic VEGF [19]. HSC-3 cells were treated with Se in serum-deprived medium (1% FBS) for 48 h. We tested the conditioned media of cells for VEGF-A production through ELISA. The inhibitory effects of selenite or MSC on VEGF expression were observed with identical patterns (Fig. 8b); however, SeMet treatment resulted in a slight increase in VEGF expression, suggesting p-Akt dependency on VEGF expression. In addition, selenite or MSC prevented HIF-1α binding activity (Fig. 8c) and its protein level (Fig. 8d) in serum-deprived HSC-3 cells, while there was no significant change by SeMet. The levels of HIF-1α protein were notably increased in $CoCl_2$-treated cells (Fig. 8d). Our results showed that HIF-1α protein accumulation and VEGF protein secretion induced by serum deprivation were obviously attenuated by Se.

Discussion

In the present study, we compared the apoptosis-inducing potential of Se compounds, namely SeMet, MSC, and selenite, against several types of solid cancer, including head and neck, lung, and breast cancers. Apoptotic induction of carcinoma cells may be a potential mechanism to mediate the anti-cancer activity of Se compounds. Although the speciation of effective metabolites of Se inside the cell is not possible at the present time, Se compounds have been shown to induce apoptosis in cancer cells in vitro with efficacy dependent on chemical forms of the original Se. Thus, we investigated the major mechanism(s) involved in Se-induced apoptosis. Consequently, each of the three was different from the other two.

Earlier studies have shown that doses (10–20 μM) of sodium selenite induce DNA single-strand breaks, reactive oxygen species, and caspase-dependent and/or caspase-independent apoptosis. Apoptosis was still induced through p53-independent pathways in this process, although wild-type p53-expressing cancer cells were more sensitive to selenite-induced apoptosis than p53-null cells [20]. In addition, selenite has been reported to induce apoptotic death in several types of cancer cells via the mitochondrial pathway [21]. These data are consistent with our observations showing that mitochondrial damage could be induced by selenite for subsequent apoptosis. In our case, analysis at a lower dose (5 μM) of selenite-treated cells showed that caspase-9 activity participated in the execution of apoptosis in carcinoma cell lines (Fig. 4a). Cancers including lung (A549) and head and neck (HSC-3) are substantially more sensitive to selenite and more prone to apoptosis induction than breast cancer (MCF-7) (Fig. 1a). This resistance may be related either to the absence of caspase-3, a caspase activated in response to apoptotic stimuli, or to deregulation of the expression of molecular chaperone (GRP94) localized in the ER [22]. This offers an opportunity for its use as a therapeutic agent if selenite can selectively induce the apoptosis of cancer cells without causing significant damage to normal cells.

Second-generation MSC directly enters the methylated pool via methylselenol in contrast to inorganic Se (selenite), which is metabolized through hydrogen selenide. MSC is activated in one step by β-lyase to the presumed active metabolite methylselenol [6]; however, because cells in culture have low levels of β-lyase, it leads to the inefficient conversion of MSC to methylselenol, and to delayed apoptosis [23]. By comparison, the effective dose of MSC used here was tenfold higher than dose of selenite. MSC induced caspase-mediated and p53-independent apoptosis (Figs. 3, 4). Several reports have documented that MSC induced apoptosis through different mechanisms

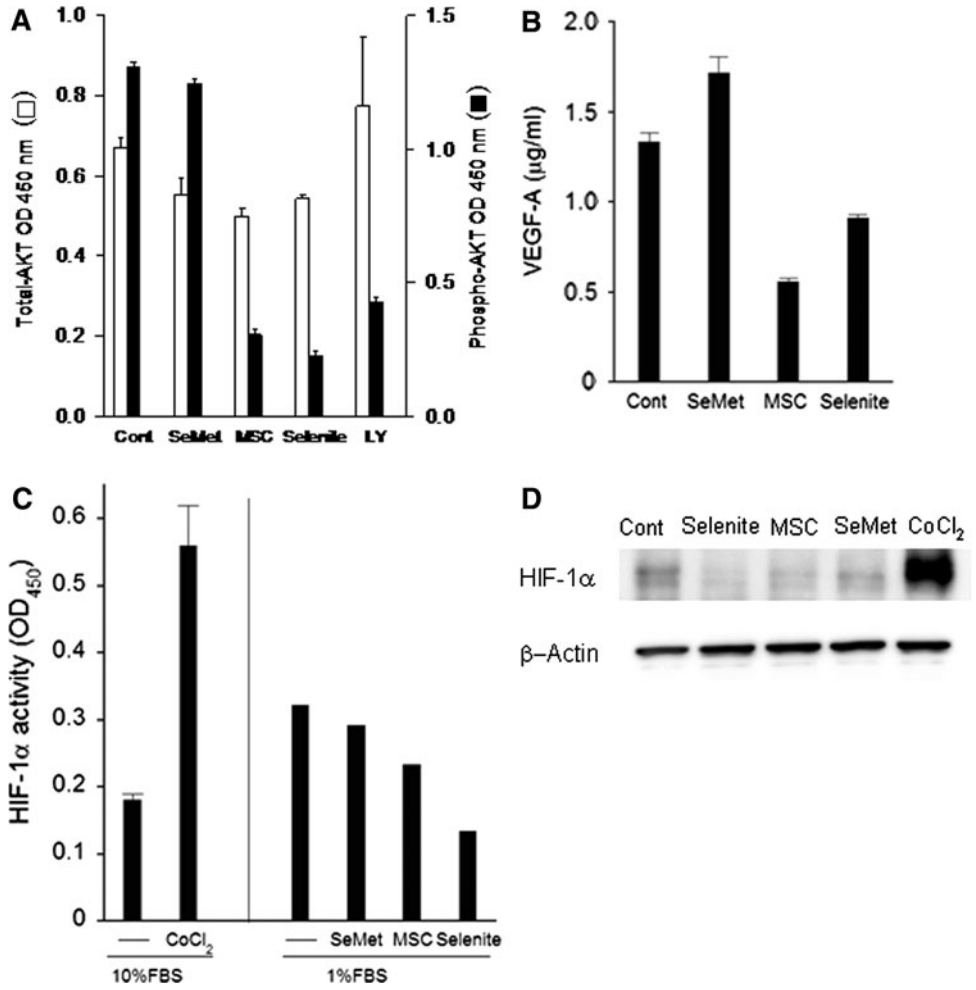

Fig. 8 Anti-angiogenic effects of Se compounds in HSC-3 cells. **a** Cells were treated with SeMet (100 μM), MSC (100 μM), selenite (10 μM) and LY294002 (LY, 30 μM) for 12 h, and fixed. Phospho-Akt and total-Akt were assayed in triplicate and reactions were measured at OD 450 nm. Results represent the mean values ± SE of OD_{450} values from triplicate experiments. **b** Cells were cultured in 10% serum medium for 24 h. After removing the medium, cells were treated with SeMet (50 μM), MSC (50 μM) and selenite (5 μM) under serum deprivation (1%) for 4 days. The conditioned medium was collected, and VEGF-A secretion was measured by ELISA. Results are the mean values ± SE of values/well from triplicate experiments. **c** Cells were treated with SeMet (100 μM), MSC (100 μM) and selenite (10 μM) in 1% serum medium for 4 h, and with $CoCl_2$ in 10% serum medium. Nuclear proteins were extracted and each sample (10 μg) was assayed for HIF-1α binding activity. **d** Cells were treated with selenite (10 μM), MSC (100 μM), SeMet (100 μM) and $CoCl_2$ (250 μM) for 4 h. Nuclear proteins were extracted for HIF-1α expression, and equal amounts of extracts were loaded for Western blotting

in various cancer lines. Caspase-3, -6, -8 and -9 were associated with the MSC-induced apoptosis of tumor cells, including cytochrome *c*-independent caspase-3 activation, the down-regulation of IAP family proteins, and Bax cleavage mediated by caspase-dependent calpain activation in ovarian cancer cells [24, 25]. In addition, MSC might down-regulate VEGF secretion in vitro through the interference of PI3K/Akt signaling regulation pathway (Fig. 8a, b). Our study provides evidence for the use of MSC as an anti-angiogenic agent, which enhances the therapeutic efficacy of a wide variety of anti-cancer agents when used in combination therapy.

Organic Se compounds, such as SeMet and MSC, are less toxic than selenite, and methylselenol is the common active metabolite. Both require enzymatic conversion to methylselenol produced by methioninase or *β*-lyase, respectively [7]. Because the level of methioninase in mammalian cells is negligible, the addition of methioninase to SeMet was essential to generate methylselenol. SeMet is poorly metabolized, and the effective dose of SeMet alone was tenfold higher than dose of selenite. Methylselenol may not be the only metabolite with important biological activity. SeMet also undergoes transamination reactions to generate α-keto-γ-methylselenobutyrate [26]. Limited information

is currently available on the molecular targets or signaling mechanisms underlying the anti-cancer effects of SeMet. Treatment with SeMet/methioninase resulted in increased levels of phosphorylated p53, total p53, Bax, and $p21^{Waf1}$ proteins. Human prostate cancer cells treated with SeMet/methioninase also showed p53 translocation to mitochondria, decreased mitochondrial membrane potential, cytochrome *c* release into the cytosol, and activation of caspase-9 [7]. The inability of apoptosis by SeMet in HSC-3 cells might be due to the absence of wild-type p53 in HSC-3 cells. In contrast, SeMet alone stimulated p53-dependent apoptotic pathways in wild-type p53-expressing A549 cells (Fig. 3a, b). PFT, which was shown to block transcriptional activity of the tumor suppressor p53 specifically [27], clearly reduced apoptosis by SeMet in A549 cells (Fig. 3c). Obviously, no reduction of p-Akt in SeMet-resistant HSC-3 cells was observed and SeMet did not affect VEGF expression and HIF-1α binding activity in the cells.

It has recently been recognized that the ER stress-induced apoptosis caused by misfolded or unfolded proteins can represent an independent mechanism of apoptosis associated with the classical apoptotic pathways induced by mitochondrial damage or death receptors [28]. The present study provides evidence to support the role of ER stress in mediating the apoptotic effect among Se compounds. Treatment with Se induced certain signature ER stress markers. Our findings of caspase-12 activation and eIF2α phosphorylation during apoptosis induced by Se in HSC-3 cells are novel as a molecular mechanism of the apoptotic activity of Se (Figs. 5a, 6). In contrast, an ER chaperone protein, BiP/GRP78, was induced modestly, but not significantly. Caspase-12, localized on the cytoplasmic side of ER, is proteolytically activated following ER stress and m-calpain activation. SJA6017 reversibly binds the active site of calpain and showed significant protective effects on SeMet-mediated cell death in A549 cells (Fig. 5b). Active caspase-12 is capable of directly activating caspase-9 and/or -3. Calpain activation therefore appears to be a crucial mechanism required for cell death in our experimental model. Maintaining p-eIF2α through Sal, an inhibitor of eIF2α dephosphorylation, can potentiate the killing of cancer cells by MSC (Fig. 7a) [29]; however, Sal clearly lacked a cytotoxic effect against the ER stress imposed by SeMet and instead enhanced the cytoprotective effect of SeMet in A549 cells (Fig. 7b) [17]. These results suggest that increased levels of p-eIF2α reflect its contribution to either apoptotic cell death or apoptotic resistance, although strong signals of p-eIF2α could not yet be detected in MSC treatment. This prompted us to more directly examine the role of eIF2α in translational initiation under Se exposure. Moreover, Se compounds may induce an atypical ER stress response using certain components of the ER stress response to induce apoptosis. The level of GRP78 is strongly elevated in a variety of cancer cell lines, correlating with protection of cancer cells against apoptotic death, higher pathologic grade, recurrence, and poor patient survival in several cancers [30]. In this study, because each Se treatment did not particularly enhance the GRP78 protein level, GRP78 was unable to plays a role in the inhibition of apoptotic signaling in cells subjected to Se-induced ER stress.

Finally, one of the most important angiogenic factors is VEGF, which initiates a number of key endothelial angiogenic responses, such as proliferation, migration, differentiation, and protection from apoptosis [31]. Most tumors express and secrete high levels of VEGF, and secretion is enhanced by hypoxia via the major hypoxia-responsive transcription activator, HIF-1α [15, 32]. In addition, VEGF-mediated pro-angiogenesis signaling via the PI3K/Akt-dependent signaling pathway enhances anti-apoptotic activity to cancer cells [33]. Consequently, HIF-1α and its downstream target VEGF are potential targets for tumor angiogenesis prevention. Although several reports have shown the effect of Se and its metabolites on VEGF expression and secretion, they have not provided any information about the effect of Se on HIF-1α/VEGF function. The VEGF expression level of HSC-3 cells treated with MSC or selenite was lower than in untreated controls, while SeMet did not reduce VEGF expression under serum deprivation (Fig. 8b). These findings were associated with its modulating effects on transcriptional factor HIF-1α (Fig. 8d). HIF-1α is known to trans-activate VEGF downstream of the PI3K/Akt pathway [34]. We believe that the anti-angiogenic effect of Se constructs a signaling complex system network, controlling the apoptotic program through the PI3K/Akt pathway.

Conclusion

In conclusion, the results reported in the present study demonstrate that Se-induced apoptosis of carcinoma cells is a caspase-dependent process that involves distinct mechanisms, respectively. Particularly relevant in this regard are activation of the intrinsic apoptotic pathway and the occurrence of ER stress, and the observed apoptosis by SeMet may be related to the p53 status. Our data indicate that Se compounds are novel drug with potential for clinical development against human malignancies.

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