Sodium Selenite Induces Superoxide-Mediated Mitochondrial Damage and Subsequent Autophagic Cell Death in Malignant Glioma Cells

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Abstract

Malignant gliomas are resistant to various proapoptotic therapies, such as radiotherapy and conventional chemotherapy. In this study, we show that selenite is preferentially cytotoxic to various human glioma cells over normal astrocytes via autophagic cell death. Overexpression of Akt, survivin, XIAP, Bcl-2, or Bcl-xl failed to block selenite-induced cell death, suggesting that selenite treatment may offer a potential therapeutic strategy against malignant gliomas with apoptotic defects. Before selenite-induced cell death in glioma cells, disruption of the mitochondrial cristae, loss of mitochondrial membrane potential, and subsequent entrapment of disorganized mitochondria within autophagosomes or autophagolysosomes along with degradation of mitochondrial proteins were noted, showing that selenite induces autophagy in which mitochondria serve as the main target. At the early phase of selenite treatment, high levels of superoxide anion were generated and overexpression of copper/zinc superoxide dismutase or manganese superoxide dismutase, but not catalase, significantly blocked selenite-induced mitochondrial damage and subsequent autophagic cell death. Furthermore, treatment with diquat, a superoxide generator, induced autophagic cell death in glioma cells. Taken together, our study clearly shows that superoxide anion generated by selenite triggers mitochondrial damage and subsequent mitophagy, leading to irreversible cell death in glioma cells. [Cancer Res 2007;67(13):6314–24]

Introduction

Gliomas account for >50% of all brain tumors and are by far the most common primary brain tumors in adults (1). Despite the use of conventional treatments, including surgery, γ-irradiation, and chemotherapy, the average life expectancy of glioma patients after the initial diagnosis is usually less than 1 year (2). Although caspase-mediated apoptosis is the best-defined cell death program counteracting tumor growth, glioma cells are resistant to the conventional proapoptotic cancer therapeutics (3). Therefore, effective treatment of malignant gliomas may rely on the development of novel strategies for inducing nonapoptotic cell death, such as autophagic cell death or cell death through mitotic catastrophe, which has been recently described as alternative death pathways (4).

Selenium, a metalloid element that occurs naturally in the earth's soil, is a key element for maintaining the activity of some antioxidant enzymes and redox-regulatory proteins (5). Several epidemiologic studies have shown an inverse association between selenium levels and cancer risks in humans (6–8), and selenium levels in the cerebrospinal fluid of patients with malignant brain tumors have been reported to be lower than those with benign tumors (9). Sodium selenite, a commonly used dietary form of selenium (10), was recently reported to confer anticancer effects through induction of apoptosis in several cancer cell types (11, 12). In this study, we show for the first time that selenite shows an anticancer effect via autophagic cell death in various glioma cells but not in astrocytes.

Autophagy, a regulated process of degradation and recycling of cellular constituents, also participates in organelle turnover and in the bioenergetic management of starvation (13, 14). During autophagy, parts of the cytoplasm or entire organelles are sequestered into double-membraned vesicles called autophagic vacuoles or autophagosomes. These autophagosomes ultimately fuse with lysosomes to generate single-membraned autophagolysosomes capable of degrading their contents. Although autophagy can serve as a protective mechanism against apoptosis and starvation by recycling macromolecules and removing damaged mitochondria and other organelles (15, 16), it can also lead to growth arrest, reduction in cell number, and a nonapoptotic cell death associated with appearance of excessive autophagic vesicles (17). Therefore, high levels of autophagy can function as a cell death effector mechanism. Recently, induction of autophagy or autophagic cell death has been reported in several types of cancer cells in response to radiation or chemotherapy (18, 19). The molecular mechanisms of autophagy remain largely unknown, including the initiating signal(s) of autophagy, the mechanism(s) by which the targeted organelle is recognized and/or selected by the autophagosome, the source(s) of the sequestering membrane, and the precise mechanism of vesicle formation.

In the present work, we present clear evidence that selenite-induced superoxide anion triggers functional and structural disruption in mitochondria, leading to mitochondria-selective autophagy (mitophagy) and subsequent nonapoptotic cell death. These findings provide novel insights into the underlying mechanisms of mitophagy, as well as offering a potential strategy for treatment of gliomas, which are resistant to proapoptotic therapeutics.

Materials and Methods

Cell culture. Human glioma cell lines U87MG, T98G, and A172 were purchased from Korean Cell Line Bank. Human glioma cell lines U343 and...
U251 were purchased from the American Type Culture Collection. Various glioma cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (Life Technologies). The primary cultures of normal human astrocytes were prepared from 14-week-gestation fetal cerebrum tissues as described previously (20). Human astrocyte cultures were grown in DMEM with high glucose supplemented with 10% FBS and 20 μg/mL gentamicin (Life Technologies) and subcultured every 2 weeks, and cell culture passage number less than five was used in the present study. Immunofluorescence study indicated that better than 99% of cells expressed glial fibrillary acidic protein, a cell type–specific marker for astrocytes. Permission to use human brain tissues for research was granted by the clinical screening committee involving human subjects of the University.

**Chemicals.** Sodium selenite, polyethylene glycol (PEG)-catalase, 3-methyladenine (3-MA), acridine orange, monodansylcadaverine (MDC), Cu(II)(diisopropylsalicylate)$_2$ (CuDIPS), Mn(III) meso-tetras (4-benzoic acid)

![Figure 1.](image)

**Figure 1.** Sodium selenite induces caspase-independent nonapoptotic cell death in various malignant glioma cells but not in normal astrocytes. A, effect of selenite on the viability of human glioma cells and astrocytes. Human glioma cells and astrocytes were treated with the indicated concentrations of selenite for 24 h. Cellular viability was assessed using calcein-AM and EthD-1. Points, mean of three independent experiments; bars, SE. * P < 0.01, compared with untreated cells. B, DNA content of glioma cells treated with selenite. U87MG cells were untreated or treated with 7 μmol/L selenite for 24 h, and DNA contents were measured by fluorescence-activated cell sorting analysis. C, inhibition of caspases by z-VAD pretreatment or CrmA overexpression does not block selenite-induced cell death. Con. cells (pcDNA3/U87MG; U87MG cells stably transfected with pcDNA3) were pretreated with 50 μmol/L z-VAD-fmk for 30 min and further treated with the indicated concentrations of selenite for 24 h. Cellular viability was assessed using calcein-AM and EthD-1. Stable U87MG cell lines overexpressing CrmA were established and CrmA expression was examined by Western blotting. Con. cells and CrmA-overexpressing U87MG cells were treated with selenite for 24 h, and cellular viabilities were compared using calcein-AM and EthD-1. Columns, average of three independent experiments; bars, SE. * P < 0.001, compared with untreated cells; ** P < 0.01, compared with cells treated with selenite. D, morphologies of selenite-treated glioma cells and astrocytes. Various glioma cells and astrocytes were treated with 7 μmol/L selenite for 12 h and observed under a phase-contrast microscope. Bar, 2 μm. Representative pictures. E, effects of the overexpression of various antiapoptotic proteins on selenite-induced cell death. Stable cell lines overexpressing Myc-tagged Akt or XIAP, Flag-tagged survivin, Flag-Bcl-2, or Bcl-xL were established and their respective overexpressions were confirmed with Western blotting using anti-Myc or anti-Flag antibodies. Con. cells and the respective overexpressing cell lines were treated with 7 μmol/L selenite for 24 h and cellular viabilities were assessed using calcein-AM and EthD-1. Columns, average of three independent experiments; bars, SE. * P < 0.01, compared with cells treated with selenite. To avoid problems of clonal variability, more than three different subclones of each overexpressing cell line were tested, and similar results were obtained in each case.
A

U87MG  T98G  U251  A172  Astrocytes

Control

Selenite

B

T98G  A172

Control  Selenite  Control  Selenite

C

0 h  6 h  12 h

D

Selenite  3-MA + Selenite

0 h  6 h  12 h  6 h  12 h

E

siRNA:

ATG6  ATG7

siRNA:

Control  ATG6  ATG7

siRNA: Control  ATG6  ATG7

% of viable cells

Selenite

3-MA (mmol/L)

0 0.25 0.5 0 0.25 0.5

% of viable cells

siRNA  Control  ATG6  ATG7
acid) porphyrin (MnTBAP), and N-acetylcysteine (NAC) were purchased from Sigma Chemical Corp. Hydroxyethidine, rhodamine 123 (Rh-123), calcein acetoxymethyl ester (calcein-AM), and ethidium homodimer (EthD-1) were purchased from Molecular Probes. Caspase inhibitor benzoxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (z-VAD-fmk) was from R&D Systems.

Measurement of cell viability. Cell viability was assessed by double staining of cells with 2 μmol/L calcein-AM and 4 μmol/L EthD-1. The calcein-positive live cells and EthD-1–positive dead cells were visualized using a fluorescence microscope (Axiovert 200M using Axiovision Release 4.4 and Axiscam HRM digital camera, Zeiss).

Establishment of the stable cell lines overexpressing CrmA, Akt, Bcl-2, Bcl-xL, XIAP, survivin, copper/zinc superoxide dismutase, manganese superoxide dismutase, or catalase and the stable cell lines expressing the fluorescence specifically in mitochondria, endoplasmic reticulum, or peroxisomes. U87MG cells were transfected with the following: a mammalian expression vector containing CrmA cDNA, a vector containing Myc-tagged active Akt (Upstate Biotechnology), a vector following: a mammalian expression vector containing CrmA cDNA, a vector containing Myc-tagged XIAP, a vector containing Flag-tagged Bcl-2, a vector containing Myc-tagged Bcl-xL, a vector containing Flag-tagged survivin, and pcDNA3 (Invitrogen). The pcDNAs encoding human copper/zinc superoxide dismutase (Cu/ZnSOD), manganese superoxide dismutase (MnSOD), and catalase were PCR amplified from plasmids containing these sequences (kindly provided by Dr. M. Akashi, National Institute of Radiological Products). Following treatment with the plasmid encoding GFP-LC3 (21), and the stable cell lines were selected with changes of medium containing 500 μg/mL G418. The stable cell lines expressing GFP-LC3 were incubated in the live cell chamber Chamälie IC (Live Cell Instrument) and then treated with 7 μmol/L sodium selenite. Translocation of GFP-LC3 from cytosol to autophagic vacuoles was observed under a fluorescence microscope equipped with Zeiss filter set #10 (excitation band pass, 450–490 nm; emission band pass, 515–565 nm), and the percentage of cells with GFP-LC3 dots was assessed.

Small interfering RNAs. The 25-nucleotide small interfering RNA (siRNA) duplexes used in this study were purchased from Invitrogen and have the following sequences: ATG6, CAGUUGGCAAAUAAUCU; ATG7, CAGAAGGAGUCACAGCUCUUCA. Cells were transfected with siRNA oligonucleotides using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's recommendations.

Measurement of mitochondrial membrane potential. Selenite-treated cells were stained with 1 μmol/L Rh-123 for 15 min, and samples were observed under a fluorescence microscope equipped with Zeiss filter set #10 (excitation band pass, 450–490 nm; emission band pass, 515–565 nm).

Transmission electron microscopy. Cells were prefixed in Karnovsky's solution [1% paraformaldehyde, 2% glutaraldehyde, 2 mmol/L calcium chloride, 0.1 mol/L cacodylate buffer (pH 7.4)] for 2 h and washed with cacodylate buffer. Postfixing was carried out in 1% osmium tetroxide and 1.5% potassium ferrocyanide for 1 h. After dehydration with 50% to 100% alcohol, the cells were embedded in Poly/Bed 812 resin (Pelco) and polished for electron microscopy. Images were collected on a transmission electron microscope (EM 902A, Zeiss).
Measurement of SOD and catalase activity. Cells \((2 \times 10^6)\) plated in 100-mm culture dishes were collected, washed with PBS, and pelleted by centrifugation at \(500 \times g\) for 10 min. The cell pellet was resuspended in ice-cold 50 mmol/L potassium phosphate (pH 7) containing 1 mmol/L EDTA, sonicated, and centrifuged at \(10,000 \times g\) for 15 min at 4°C. The supernatant fraction was used for determination of SOD or catalase activity using kits from Cayman Chemical Co. according to the manufacturer's instructions.

Statistical analysis. All data are presented as mean \(\pm\) SE of at least three independent experiments. The statistical significance of differences was assessed using ANOVA (GraphPad Software) followed by Student-Newman-Keuls' multiple comparison tests. \(P < 0.05\) was considered significant.

Results

Sodium selenite induces nonapoptotic cell death in human glioma cells. We examined the effect of sodium selenite on viability in a variety of glioma cells and normal astrocytes. Our results revealed that 1 to 7 \(\mu\)mol/L selenite decreased viability in the tested glioma cell lines, whereas the human astrocytes were relatively resistant to the same doses (Fig. 1A), suggesting that selenite is preferentially cytotoxic to malignant glioma cells over normal astrocytes. We next examined whether the cytotoxic effect of selenite on glioma cells is associated with induction of apoptosis. Flow cytometric analysis of the cellular DNA content showed that the sub-G1 population was not increased in U87MG cells treated with 7 \(\mu\)mol/L selenite for 24 h (Fig. 1B), although cellular viability decreased up to 20% at this time point (Fig. 1A). When we further assessed DNA fragmentation by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay, TUNEL-positive cells were rarely found in T98G cells treated with 7 \(\mu\)mol/L selenite for 24 h (Supplementary Fig. S1). Selenite-induced cell death was not inhibited by pretreatment with the pan-caspase inhibitor z-VAD or by overexpression of the viral caspase-8 inhibitor CrmA (Fig. 1C; ref. 23). Selenite-treated glioma cells showed numerous small vesicles around their nuclei before selenite-induced cell death, whereas treated astrocytes did not (Fig. 1D). However, no apoptotic morphologies, such as cellular shrinkage, formation of apoptotic bodies, membrane blebbing, and nuclear fragmentation, were observed during selenite-induced cell death. Malignancy and resistance to various conventional anticancer therapies of gliomas have been associated with Akt activation due to mutations in the PTEN pathway and high expression of XIAP or survivin (24–26). In addition, Bcl-2 and Bcl-xL have been shown to protect various cancer cells from death stimuli (27). Accordingly, we investigated whether overexpression of these antiapoptotic proteins could attenuate selenite-induced cell death but found no evidence that selenite-induced cell death was affected by any of them (Fig. 1E). Taken together, selenite induces nonapoptotic cell death in human glioma cells but not in normal human astrocytes, suggesting that selenite treatment may provide an effective therapeutic strategy for malignant gliomas, many of which are resistant to conventional treatments due to inherent defects in apoptotic machineries.
Selenite induces autophagy in glioma cells. We investigated whether the vacuolation observed in selenite-treated glioma cells was associated with autophagy. To identify the development of AVOs, which are characteristic of autophagy (19), we used the lysosomotropic agent acridine orange. Staining with acridine orange followed by fluorescence microscopy revealed significant development of AVOs beginning 6 h after treatment in U87MG, T98G, U251, and A172 glioma cells, but no AVO development was observed in astrocytes by 16 h after treatment (Fig. 2A). To further assess the possibility of autophagy, we stained sodium selenite–treated and untreated glioma cells with MDC, an autofluorescent base that has been reported to accumulate in autophagic vacuoles (28). Fluorescence microscopy revealed that the sodium selenite–treated T98G and A172 cells showed high-intensity accumulation of MDC into granular structures, whereas untreated glioma cells did not (Fig. 2B). Vesicular accumulation of LC3 (microtubule-associated protein 1 light chain 3) is a marker of autophagy, as recruitment of modified LC3 to the nascent autophagic vesicle is a common event in autophagosome formation (21). To reliably detect the formation of autophagic vesicles, we expressed GFP-tagged LC3 (21) in U87MG cells and examined changes in the expression of GFP-LC3 following selenite treatment. In untreated cells, GFP-LC3 showed diffuse expression, whereas in selenite–treated cells it showed ring-shaped pattern around the vacuoles at 6 h and a high-intensity punctuate expression at 12 h (Fig. 2C). Similar results were obtained in T98G cells stably transfected with GFP-LC3 (Fig. 2D, a). The functional role of autophagy in selenite–induced cell death was investigated using 3-MA, the specific inhibitor of early stages of the autophagic process (29). Pretreatment of GFP-LC3–transfected U87MG cells with 3-MA attenuated

Figure 4. A, electron microscopic observation of glioma cells treated with selenite. T98G cells were untreated or treated with 7 μmol/L selenite for the indicated times and transmission electron microscopy was done. Bar, 2 μm. B, mitochondria (Mito.) and autophagic vacuoles may fuse after selenite treatment. YFP-Mito cells were untreated or treated with 7 μmol/L selenite for 12 h and then incubated with MDC. Changes in mitochondrial morphologies and cellular MDC uptake were observed under a fluorescence microscope. Bar, 2 μm. C, mitochondria and lysosomes (Lyso.) may fuse after selenite treatment. YFP-Mito cells were left untreated or treated with 7 μmol/L selenite for 12 h and then incubated with LysoTracker Red. Changes in mitochondrial morphologies and lysosomes were observed under a fluorescence microscope. Bar, 2 μm. D, reduction of mitochondrial protein levels during selenite-induced cell death. T98G, U87MG, or U343 cells were treated with 7 μmol/L selenite for the indicated time points, lysed, and subjected to Western blotting for the indicated mitochondrial proteins. Equal loading of protein samples was confirmed by Western blotting using α-tubulin antibody.
Selenite-induced translocation of GFP-LC3 and cell death (Fig. 2D, a and b). Furthermore, siRNA-mediated knockdown of either ATG6 or ATG7 expression also inhibited the translocation of GFP-LC3 and cell death induced by selenite (Fig. 2E), suggesting the involvement of ATG6 and ATG7 in selenite-induced autophagic cell death. Taken together, these results show that excessive autophagy induced by selenite contributes to nonapoptotic cell death.

**Selenite-damaged mitochondria are targeted by autophagy.** Because mitochondria play a crucial role in regulating cell death, we next examined whether selenite-induced autophagic cell death in glioma cells was associated with alterations in mitochondrial function and/or structure. Measurement of mitochondrial membrane potential (MMP) using Rh-123 showed that treatment of T98G cells with 7 μmol/L selenite induced a significant loss of MMP beginning 4 h after treatment (Fig. 3A). Interestingly, following exposure to selenite, decreased MMP was always detected in vacuolated cells. We then examined selenite-induced changes in mitochondrial morphology using an U87MG subline stably transfected with pEYFP-Mito plasmid (YFP-Mito cells). As shown in Fig. 3B, mitochondria in untreated YFP-Mito cells showed filamentous or elongated morphologies but became fragmented within 2 h after selenite treatment. Notably, all mitochondria exactly colocalized with the numerous small vacuoles 6 h after treatment. Beyond 6 h after treatment, the numbers and sizes of vacuoles expressing mitochondria-specific fluorescence increased further. These results suggest the possibility that fragmented mitochondria by selenite treatment may be engulfed by autophagic vacuoles. To test whether the autophagic vacuoles also colocalized with the ER or peroxisomes during selenite-induced autophagy, we used a stable U87MG subline transfected with pEYFP-ER or pEGFP-Peroxi plasmid. In contrast with mitochondria, colocalization of the vacuoles with the ER or peroxisomes was minimal in selenite-treated cells, suggesting that mitochondria may be selectively targeted during selenite-induced autophagy. Transmission electron microscopy to observe ultrastructural changes showed that the mitochondria appeared as highly branched and interconnected tubular networks in untreated T98G cells (Fig. 4A, a and b). Two hours after treatment with selenite, however, the mitochondria appeared shorter and more rounded and the cristae were dramatically disrupted (Fig. 4A, c and d). From 4 h after treatment, swollen mitochondria filled with an electron-dense material, presumably mitochondrial remnants, were frequently observed (Fig. 4A, e-j, black arrowheads). Furthermore, these disorganized mitochondria were frequently surrounded by the double membrane associated with autophagosomes (Fig. 4A, e-j and i, black arrows). At 8 h after treatment, more advanced mitochondrial disruption was observed with frequent fusion between the vacuoles appearing to increase their sizes (Fig. 4A, h and i) and we observed ongoing fusion of autophagosomes with lysosomes (Fig. 4A, j, white arrowheads). Staining of selenite-treated...
Figure 6. Selenite-induced autophagic cell death is blocked by overexpression of Cu/ZnSOD or MnSOD but not by catalase. A, a, establishment of the stable cell lines overexpressing antioxidant proteins. U87MG cells were stably transfected with expression vectors encoding Cu/ZnSOD, HA-MnSOD, or HA-catalase. As a control, Con. cells (U87MG cells transfected with pcDNA3) were used. Protein levels of the respective antioxidant proteins were analyzed by Western blotting using anti-Cu/ZnSOD or anti-HA antibodies, and equal loading of protein samples was confirmed by Western blotting of α-tubulin. b, Measurement of SOD and catalase (CAT) activity in the established stable cell lines. SOD activity in the lysates from the stable cell lines overexpressing Cu/ZnSOD or MnSOD was measured and compared with that of Con. cells using SOD assay kit (Cayman Chemical Co.). Representative results of three independent experiments. Catalase activity in the lysates from the stable cell lines overexpressing catalase was also compared with that of Con. cells using catalase assay kit (Cayman Chemical Co.). Similar results were obtained from three independent experiments. B, overexpression of SOD, but not catalase, blocks selenite-induced cell death. Con. cells and cells overexpressing Cu/ZnSOD, MnSOD, or catalase were treated with 7 μmol/L selenite for 24 h, and cellular viabilities were assessed using calcine-AM and EthD-1. Columns, average of three independent experiments; bars, SE. *, $P < 0.001$, compared with cells treated with selenite. C, overexpression of Cu/ZnSOD or MnSOD, but not catalase, blocks selenite-induced loss of MMP and autophagy. Con. cells and cells overexpressing Cu/ZnSOD (clone 15), MnSOD (clone 5), or catalase (clone 25) were treated with 7 μmol/L selenite for 8 h and then incubated with Rh-123. Representative fluorescent and phase-contrast images. Bar, 2 μm (top). The same cells were incubated with 7 μmol/L selenite for 12 h and then stained with acridine orange. Representative fluorescent and phase-contrast images. Bar, 2 μm (bottom). D, generation of superoxide by diquat induces autophagy. For detection of diquat-induced superoxide generation, T98G cells were treated with or without 10 μmol/L diquat for 12 h and then stained with hydroethidine. Representative fluorescent and phase-contrast images. Bar, 2 μm (D, a). For the detection of MMP loss or induction of autophagy, T98G cells were treated with or without 10 μmol/L diquat for 16 h and then stained with Rh-123, acridine orange (AO), or MDC. Representative fluorescent and phase-contrast images. Bar, 2 μm (D, b–d).
YFP-Mito cells with MDC or LysoTracker Red showed that the mitochondria significantly overlapped with the autophagic vacuoles and lysosomes, respectively (Fig. 4B and C). In addition, Western blot analysis revealed that the total amounts of mitochondrial proteins, such as cytochrome c, Smac, and OxPhos Complex II (succinate ubiquinol oxidoreductase) 70-kDa subunit, gradually decreased following selenite treatment (Fig. 4D). These results suggest that selenite-damaged mitochondria may ultimately be degraded via fusion with autophagosomes and lysosomes. Taken together, selenite may damage preferentially mitochondria in treated glioma cells, which then undergo autophagy to remove the disrupted mitochondria. However, excessive mitophagy may result in irreversible cell death.

**Selenium-induced superoxide generation triggers mitochondrial damage and subsequent autophagic cell death.** Next, we investigated the upstream regulatory mechanisms leading to selenite-induced mitochondrial damage and subsequent autophagic cell death. Selenite has been reported to increase the generation of superoxide anion during apoptosis (30, 31). Thus, we tested whether intracellular superoxide levels increase during selenite-induced autophagy in glioma cells. Hydroethidine-based measurements (22) revealed that intracellular superoxide levels were significantly increased in T98G cells from 2 h of selenite treatment compared with untreated cells (Fig. 5A). Similar trends were observed in luminometric analysis to detect superoxide levels using lucigenin (30) and H$_2$DCFDA-based detection of total reactive oxygen species (ROS) by fluorescence microscopy (data not shown). To examine whether superoxide plays a role in selenite-induced autophagic cell death, we pretreated T98G cells with various antioxidants and then exposed the cells to 7 μmol/L selenite for 24 h. Selenite-induced cytotoxicity was significantly blocked by pretreatment with a SOD mimetic CuDIPS or MnTBAP, whereas pretreatment with PEG-catalase could not block selenite-induced cell death (Fig. 5B). Pretreatment with NAC also showed a considerable blocking effect on selenite-induced cell death. Consistent with this finding, selenite-induced loss of MMP in T98G cells was significantly blocked by CuDIPS, MnTBAP, or NAC but not by PEG-catalase (Fig. 5C). Moreover, selenite-induced AVO formation was almost completely inhibited by CuDIPS, MnTBAP, or NAC but not by PEG-catalase (Fig. 5D). SOD is able to reduce the superoxide radical into hydrogen peroxide (H$_2$O$_2$). Catalase catalyzes decomposition of H$_2$O$_2$ to ground-state O$_2$. Because SOD mimetics and PEG-catalase showed different effects on selenite-induced autophagic cell death, we further examined the effect of SOD or catalase by establishment of U87MG sublines stably overexpressing Cu/ZnSOD, MnSOD, or catalase (Fig. 6A, a). Overexpression of Cu/ZnSOD or MnSOD in U87MG cells resulted in the enhancement of SOD activity. In U87MG cells stably overexpressing catalase, increased catalase activity was measured (Fig. 6A, b). Interestingly, selenite-induced cell death was significantly blocked by overexpression of Cu/ZnSOD or MnSOD but not by catalase (Fig. 6B). Very similar results were obtained using retroviral vector system overexpressing Cu/ZnSOD, MnSOD, or catalase (Supplementary Fig. S2), clearly showing that the regulatory roles of SOD and catalase in selenite-induced cell death are different. Selenite-induced dissipation of MMP and development of AVOs were also blocked by overexpression of Cu/ZnSOD or MnSOD but not by catalase (Fig. 6C). Moreover, treatment of T98G cells with diquat, a superoxide generator, induced autophagy in a manner similar to selenite (Figs. 2A and B, 3A, and 5A), including increased superoxide levels (Fig. 6D, a), MMP loss (Fig. 6D, b), and the formation of autophagic vacuoles (Fig. 6D, c and d). In contrast, treatment of T98G cells with various doses of H$_2$O$_2$, nitric oxide donor SNAP, or peroxynitrite donor SIN-1 did not induce any evidences of autophagy (data not shown). Taken together, these results show that superoxide generation plays a major role in selenite-induced mitochondrial damage and subsequent autophagy leading to irreversible cell death.

**Discussion**

Selenium is an essential trace element with potent chemopreventive effects against various cancers (5). Sodium selenite is a common dietary form of selenium (10). To date, research into the cytotoxic effects of selenite in various cancer cells has predominantly focused on its apoptosis-inducing effects (12, 30, 32). Interestingly, we herein show that treatment with selenite can effectively induce autophagy and subsequent nonapoptotic cell death in malignant glioma cells. Autophagy was traditionally believed to be a random, nonselective process through which cytoplasm and organelles are sequestered into autophagosomes and delivered to lysosomes for degradation. However, a new concept of selective autophagy has emerged in recent years. The identification of the yeast mitochondrial protein Uth1P, which is required for mitochondrial autophagy (mitophagy) but not for macroautophagy, provided evidence that mitochondria can be specifically targeted for degradation by autophagy (33). However, few previous reports have described selective removal of an entire cohort of mitochondria via autophagy in mammals (34, 35). In the present study, high-power transmission electron microscopy images show that selenite treatment induced rapid disruption of cristae and mitochondrial swelling. Decreased cellular retention of Rh-123 indicated that a mitochondrial permeability transition (MPT) was induced by selenite treatment. Fluorescence microscopic analysis using YFP-Mito cells, YFP-ER, or GFP-Peroxi cells and transmission electron microscopy showed that autophagic vacuoles subsequently entrapped the disorganized mitochondria preferentially over other organelles, such as the ER or peroxisomes. Furthermore, various mitochondrial proteins were degraded in the progression of selenite-induced autophagy. Collectively, these results show that selenite induces excessive mitophagy. It is not yet known how the mitochondria are specifically selected for autophagy following exposure to selenite. MPT has been proposed to trigger the engulfment of depolarized mitochondria by autophagy (36). Alternatively, oxidatively damaged proteins or lipids on the surface of the mitochondria in selenite-treated cells may provide an “eat me” signal, mediating digestion by highly active autophagic vacuoles. It is interesting to speculate on whether autophagic depletion of mitochondria is the direct cause of selenite-induced cell death. We presume that selenite-enhanced autophagic activity and subsequent removal of a cohort of mitochondria might contribute to irreversible cell death perhaps because a certain number of functional mitochondria are required for energy production and cell survival. Alternatively, selenite-induced autophagy of mitochondria may hasten the progression of nonapoptotic cell death by eliminating a major source of ATP production and decreasing the release of mitochondria-derived proapoptotic factors, thus tipping the cellular balance away from apoptosis.

In terms of the mechanism by which selenite treatment affects the mitochondria, our results indicate that selenite-induced intracellular superoxide generation acts to trigger mitochondrial damage and subsequent autophagic cell death. Several lines of
Mitophagy and Subsequent Cell Death by Superoxide

Mitochondria, the main site of cellular respiration, are known to play a crucial role in regulating cell survival and death. They are sensitive to oxidative stress, and their dysfunction can lead to cell death. Superoxide, a reactive oxygen species (ROS), is known to play a role in the regulation of cell death. In the context of the mitochondrial electron transport chain, superoxide generated by complex I can trigger mitochondrial damage and subsequent autophagic cell death.

Evidence support this hypothesis. First, selenium-induced superoxide anion generation was accompanied by mitochondrial damage, including MMP dissipation, disruption of cristae, and fragmentation/swelling of mitochondria. Second, not only pretreatment with CuDTP or MnTBAP but also overexpression of Cu/ZnSOD or MnSOD significantly blocked selenite-induced mitochondrial damage, indicating that superoxide is a critical mediator of mitochondrial damage and subsequent autophagic cell death [32].

Furthermore, H2O2, SIN-1, or SNAP treatment failed to induce autophagic cell death, suggesting that selenite-induced autophagic cell death might be due to its strong scavenger activity against ROS, including superoxide anion [32]. Zhao et al. (32) recently showed that generation of superoxide in gliomas is not dependent on p53. However, we found that selenite-induced superoxide generation and autophagic cell death occurred in glioma cells, whereas treatment with diquat induced autophagic cell death in these cells. These results suggest that superoxide, rather than other types of ROS, is responsible for selenite-induced mitochondrial damage and subsequent autophagy. A considerable protective effect of NAC on selenite-induced autophagic cell death might be due to its strong scavenger activity against ROS, including superoxide anion [32].

In summary, the present results show that the elevated levels of intracellular superoxide generated by selenite treatment of glioma cells initiated an autophagic cell death in which mitochondrial damage served as the main target. These novel findings not only improve our understanding of the autophagic effect of selenite and the role of superoxide in this process but also offer a potential therapeutic strategy for malignant gliomas, which are resistant to various proapoptotic therapeutics.

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