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Original Contribution

Selenite induces redox-dependent Bax activation and apoptosis in colorectal cancer cells

Fang Huang [a], Chunlai Nie [b,c], Yang Yang [a], Wen Yue [b,c], Yun Ren [a], Yingli Shang [b,c], Xiaohui Wang [b,c], Haijing Jin [b,c], Caimin Xu [a,*], Quan Chen [b,c,*]

[a] National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100005, China
[b] Joint Laboratory of Apoptosis and Cancer Biology, College of Life Sciences, Nankai University, Tianjin 300071, China
[c] State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China

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ABSTRACT

Emerging evidence suggests that selenium has chemotherapeutic potential by inducing cancer cell apoptosis with minimal side effects to normal cells. However, the mechanism by which selenium induces apoptosis is not well understood. We have investigated the role of Bax, a Bcl-2 family protein and a critical regulator of the mitochondrial apoptotic pathway, in selenite-induced apoptosis in colorectal cancer cells. We found that supranutritional doses of selenite could induce typical apoptosis in colorectal cancer cells in vitro and in xenograft tumors. Selenite triggers a conformational change in Bax, as detected by the 6A7 antibody, and leads to Bax translocation into the mitochondria, where Bax forms oligomers to mediate cytochrome *c* release. Importantly, we show that the two conserved cysteine residues of Bax seem to be critical for sensing the intracellular ROS to initiate Bax conformational changes and subsequent apoptosis. Our results show for the first time that selenite can activate the apoptotic machinery through redox-dependent activation of Bax and further suggest that selenite could be useful in cancer therapy.

Selenium is widely regarded as a protective agent against cancer risks. Supranutritional levels of selenium have benefits in preventing several types of cancer, including lung cancer, colorectal cancer, and prostate cancer. The chemopreventive role of selenium is well supported by epidemiological, preclinical, and clinical evidence [1–3]. Moreover, there is emerging evidence indicating the potential of selenium compounds in cancer chemotherapy [4]. Among the proposed anti-cancer mechanisms, cell growth inhibition and apoptosis were postulated to be critical [3,5]. Previous work, including that of our group, showed that selenite activates stress-related signaling pathways, including JNK and ERK, leading to an apoptotic cascade [6–8]. Selenite, when diffused into the cytoplasm, could perturb the intracellular redox status by reacting with intracellular thiols. In the presence of thioredoxin reductase or the thiol group, it is reduced into selenide, which cycles continuously to generate ROS in the presence of thiols and O_2 [5]. Indeed, the perturbation of the redox system and subsequent ROS generation were found to be causally linked to cancer cell death induced by selenite [9]. However, the precise mechanisms by which selenium activates the apoptotic machinery remain poorly understood.

Bcl-2 family proteins play a central role in the regulation of cytochrome *c* release and apoptosis. This family includes antiapoptotic proteins such as Bcl-2 and Bcl-xL and "multidomain" proapoptotic proteins such as Bax and Bak [10,11]. Despite the presence of the transmembrane domain, Bax is found in the cytosol, where it maintains an inactive form. In response to apoptotic signals, Bax alters its conformation, exposing its C-terminal membrane-anchoring domain and inserting into mitochondrial membranes [12]. Oligomerization of Bax or Bak at the mitochondrial membrane mediates the release of cytochrome *c* and other apoptogenic proteins into the cytosol [13]. Cells deficient in Bax are largely resistant to apoptosis induced by a number of death stimuli [14], further highlighting its critical role in apoptosis regulation. We recently reported that the cysteine residues in Bax can chemically react with ROS, leading to a change in its conformation and subsequent activation [16]. Here we investigate the molecular details of how Bax is activated by selenite. Our results reveal that the two conserved cysteine residues seem to be critical for sensing the intracellular ROS and to initiate Bax conformational changes and subsequent apoptosis. We further highlight the potential chemotherapeutic effects of selenite.

Abbreviations: MnTMPyP, manganese(III) tetrakis(*N*-methyl-2-pyridyl)porphyrin; DAPI, 4′,6-diamidino-2-phenylindole; DTT, dithiothreitol; NAC, *N*-acetylcysteine; BSO, buthionine sulfoximine; DSS, disuccinimidyl suberate; MPB, 3-(*N*-maleimidopropionyl) biocytin.

* Corresponding authors. Joint Laboratory of Apoptosis and Cancer Biology, College of Life Sciences, Nankai University, Tianjin 300071, China.
E-mail addresses: caiminxu@yahoo.com.cn (C. Xu), chenquan@nankai.edu.cn (Q. Chen).

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Materials and methods

Chemicals

H_2O_2, *N*-acetyl-L-cysteine (NAC), buthionine-sulfoximine (BSO), and anti-actin (A-5411) and anti-Bax 6A7 monoclonal antibodies were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Manganese (III) tetrakis(*N*-methyl-2-pyridyl)porphyrin (MnTMPyP) was purchased from Alexis Biochemicals (San Diego, CA, USA). Disuccinimidyl suberate (DSS) was obtained from Pierce (Rockford, IL, USA). Anti-GFP (B-2, sc-9996) monoclonal and anti-Bax (N-20, sc-493) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-cytochrome *c* and anti-Bcl-xL antibodies were from BD Biosciences (San Jose, CA, USA). CM-H_2DCFDA and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Molecular Probes (Eugene, OR, USA). The CaspACE FITC-VAD-FMK in situ marker was purchased from Promega (Madison, WI, USA).

Plasmids

The mammalian expression vector encoding human Bax (pEFGP-C3-Bax) was obtained from R.J. Youle (National Institutes of Health, Bethesda, MD, USA). Bcl-xL was inserted into pCDNA 4-TO/B. The Bax site-directed mutant constructs were made using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The following primers were used to generate the site-directed mutant constructs used in this study: Cys/Ser62 (C62/S) sense, 5′-CACCAAGAAGCTGAGC-GAGTCTCTCAAGCGCATCGGGGACG-3′; Cys/Ser126 (C126/S) forward, 5′-GGCCCTGTGCACCAAGGTCCCGGAACTGATCAGAACC-3′.

Cell culture and transfection

SW480, HCT116, and mouse embryonic fibroblast (MEF) cells were grown in DMEM supplemented with 10% FBS (Hyclone) and 1% penicillin–streptomycin at 37 °C under 5% CO_2. To establish Bax-stable transfectants, SW480 cells were transfected with pEGFP-C3-Bax and Bax mutant plasmid DNA, and positive clones overexpressing Bax were selected with 1 mg/ml G418 as described previously [16].

Immunofluorescence microscopy

Cells were grown to 70% confluence on a coverslip. After treatment, the cells were washed twice with PBS and then fixed with freshly prepared 3.7% formaldehyde at 37 °C for 15 min; 0.2% Triton X-100 was used to increase antigen accessibility. The cells were then incubated with an anti-cytochrome *c* antibody for 1 h and, after being washed, were stained with Texas red-conjugated goat anti-mouse IgG antibody. Cell images were captured with an LSM 510 Zeiss confocal microscope. DAPI was used to identify the nucleus.

Subcellular fractionation

Subcellular fractionation was performed as described previously [15,16]. Briefly, cells were harvested and resuspended in hypotonic buffer. After gentle homogenization with a Dounce homogenizer, cell lysates were subjected to differential centrifugation. The resulting membrane fractions were lysed and subjected to Western blot analysis.

Detection of Bax conformational change

The assay to detect a conformational change in Bax was performed as described previously [15,16]. Briefly, cells were lysed with Chaps lysis buffer (10 mM Hepes (pH 7.4), 150 mM NaCl, 1% Chaps, protease inhibitors). Protein samples were incubated with an anti-Bax 6A7 monoclonal antibody and then with protein G–agarose. The beads were washed three times in Chaps buffer, boiled in loading buffer, and subjected to Western blot analysis.

Cross-linking of Bax protein

Cross-linking of the Bax protein was performed as described previously [15,16]. Briefly, cells were harvested and resuspended in conjugating buffer (150 mM NaCl, 20 mM Hepes (pH 7.2), 1.5 mM $MgCl_2$, 10 mM glucose). The samples were incubated with 2 mM DSS at room temperature for 30 min. DSS was then quenched by Tris–HCl (pH 7.5). The samples were lysed and subjected to Western blot.

SDS–PAGE and Western blot

SDS–PAGE and Western blot were performed as described previously [15,16]. Briefly, the cells or the membrane fractions were lysed in lysis buffer (10 mM Hepes, pH 7.4, 2 mM EGTA, 0.5% NP-40, protease inhibitors). Equivalent samples (20 μg protein) were subjected to SDS–PAGE and then transferred onto nitrocellulose membranes. Membranes were probed with the indicated antibodies followed by appropriate HRP-conjugated secondary antibodies (KPL, Gaithersburg, MD, USA). Immunoreactive bands were visualized with a chemiluminescence kit (Pierce).

3-(N-maleimidopropionyl)biocytin (MPB) labeling and purification

Cells were harvested and lysed with lysis buffer (0.5% NP-40, 25 mM Hepes (pH 7.7), 5 mM EDTA, protease inhibitors), and 40 mM *N*-ethylmaleimide (NEM; Sigma) was added to block free thiols, which could undergo redox modification upon cell lysis. Protein samples were assayed for concentration using the Bradford method and precipitated with ice-cold acetone. The air-dried pellet was dissolved in denaturing buffer (25 mM Hepes (pH 7.7), 2 mM EDTA, 2.5% SDS) containing 40 mM NEM and incubated for 60 min at 50 °C to block free thiols completely. The samples were precipitated with ice-cold acetone, washed with 80% acetone to remove excess NEM, and then dissolved in denaturing buffer containing 10 mM dithiothreitol (DTT), incubated for 30 min at 50 °C, acetone-precipitated, and acetone-washed to remove excess DTT. The resulting pellets were dissolved in denaturing buffer containing 0.5 mM MPB (Invitrogen) and incubated for 60 min at 30 °C in the dark. Extracts were acetone-precipitated to remove unbound MPB, dissolved in denaturing buffer, and incubated overnight at 4 °C with continuous tumbling in binding buffer (25 mM Hepes (pH 7.7), 2 mM EDTA, 0.8% SDS, 0.3% Triton X-100, 70 mM NaCl) containing streptavidin–agarose beads (Pierce). The resin was washed five times with washing buffer (25 mM Hepes (pH 7.7), 2 mM EDTA, 0.5% Triton X-100, 600 mM NaCl), boiled in SDS–PAGE loading buffer for 3 min, and subjected to Western blot analysis. A negative control was used to demonstrate that free thiol blocking was sufficient: a sample was reduced with 10 mM DTT for 30 min at 50 °C and then subjected to the procedure above, with the exception of the DTT reducing step before MPB labeling.

Estimation of free thiol and GSH levels

The total amount of SH groups in the cell homogenate or GSH levels after precipitation of the protein from the samples was assayed through the addition of 50 μl of DTNB (0.4 mg/ml) and 500 μl of 6 M guanidine–HCl in 0.2 M Tris–HCl, pH 8.0, to an aliquot of homogenate in a 1-cm cuvette. The absorbance at 412 nm was measured after 5 min and the concentration was calculated using $\varepsilon_{412} = 13.6/\text{mM}$.

Tumor models in vivo

SW480 colon cancer cells (2×10^6) were inoculated subcutaneously in 5-week-old female nu/nu mice at the right back. Five mice

were used per group. After 1 week, when the tumors were palpable, selenite was dissolved in PBS and given intraperitoneally (2 mg/kg/day). The control group received vehicle only (PBS). Tumor dimensions were measured with calipers and the volume was calculated using the following formula: volume $= 0.5 \times l \times w^2$, with l being the maximal length and w being the width. All the mice were maintained and treated according to the *UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia.*

Statistical analysis

In all the quantitative analyses represented in histograms using cultured cells, the values are expressed as means ± SD and were derived from three independent experiments. In the quantitative analyses using in vivo tumor models and tumor specimens, statistical analysis was performed using Student's t test, with p values <0.05 considered significant; five tumor specimens from the five mice in each group were used, and at least 500 cells were counted in each specimen.

Results

Selenite induces apoptosis and Bax activation in colon cancer cells

We tested the sensitivity of three colon cancer cell lines (SW480, HCT116, and HT29) to supranutritional doses of selenite and found that, when administered at or above 5 μM, typical apoptotic hallmarks could be observed. Annexin V staining showed that 10 μM selenite induced a significant increase in the apoptotic population in these cells (Fig. 1A), and Hoechst staining demonstrated that selenite induced typical nucleus fragmentation (Fig. 1B). In addition, intracellular caspase was activated by selenite in these cells as measured by CaspACE FITC-VAD-FMK staining (Fig. 1C). These results suggest that supranutritional doses of selenite could induce typical apoptosis in various colon cancer cell lines. We compared the sensitivity of primary cultured cells derived from human embryo intestine epithelial cells with SW480 cells and found that SW480 cells are more sensitive to selenite than normal cells (Fig. 1D). Our previous studies showed that selenite induces mitochondria-dependent apoptosis in leukemia cells

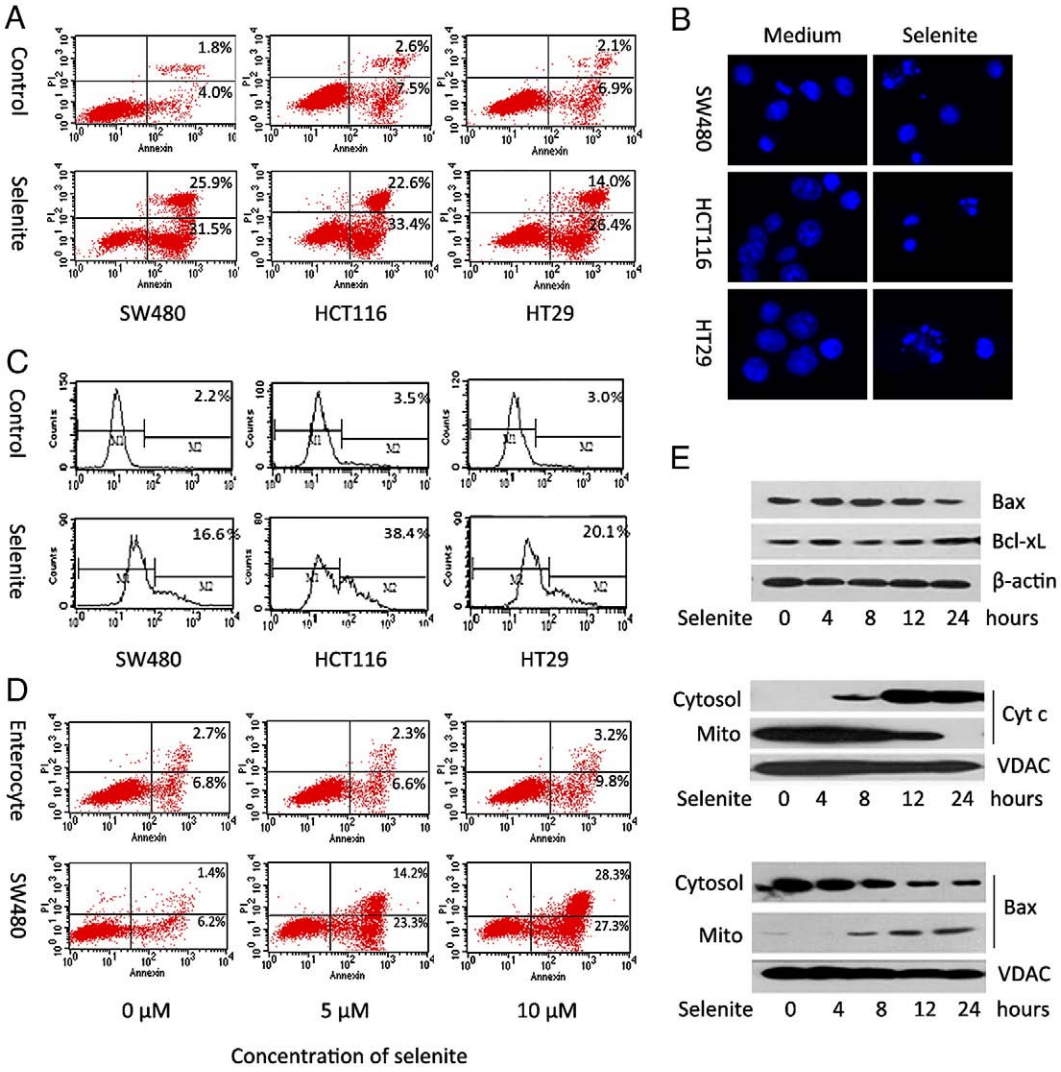

Fig. 1. Selenite induces apoptosis and Bax activation in colon cancer cells. (A) Flow-cytometric analysis of cell apoptosis by annexin V and propidium iodide double staining. SW480, HCT116, and HT29 colon cancer cells were treated with 10 μM selenite for 24 h. (B) Hoechst staining showing nucleus fragmentation, a morphological hallmark of apoptosis. SW480, HCT116, and HT29 colon cancer cells were treated with 10 μM selenite for 24 h. (C) Flow-cytometric analysis of caspase activity by CaspACE FITC-VAD-FMK staining. SW480, HCT116, and HT29 colon cancer cells were treated with 10 μM selenite for 24 h. (D) SW480 cells and primary cultured cells derived from human embryo intestine epithelial cells were treated with 0, 5, or 10 μM selenite for 24 h and subjected to apoptosis analysis. (E) Upper: Western blot analysis of Bax and Bcl-xL expression levels and lower: subcellular fractionation demonstrating cytochrome c and Bax translocation. SW480 cells were treated with 10 μM selenite for indicated durations. All data are representative of three independent experiments.

[17], and consistently, we found that selenite induces cytochrome *c* release in SW480 colon cancer cells (Fig. 1E).

We next examined the changes in Bcl-2 family proteins, which are central regulators of the mitochondrial apoptotic pathway. Bax expression levels were up-regulated 4 h after selenite treatment (Fig. 1E), before cytochrome *c* release, whereas there were no significant changes in the expression of the antiapoptotic member Bcl-xL. Moreover, cell fractionation experiments showed that the protein level of Bax decreased in the cytosolic fraction and concomitantly increased in the mitochondria-enriched heavy membrane fractions of SW480 cells starting at 8 h after selenite treatment (Fig. 1E). These results suggest that, similar to arsenic trioxide [16], supranutritional doses of selenite could induce Bax activation and activate the intrinsic apoptotic pathway in colon cancer cells.

Selenite induces Bax translocation, conformational change, and oligomerization in a Bcl-xL-inhibitable manner

We next asked how selenite promoted Bax translocation from the cytosol onto mitochondria. To closely monitor the process of Bax translocation and its consequences for apoptosis, SW480 cells stably expressing the cDNA of wild-type full-length human Bax fused to green fluorescent protein (GFP) (and the empty vector) were

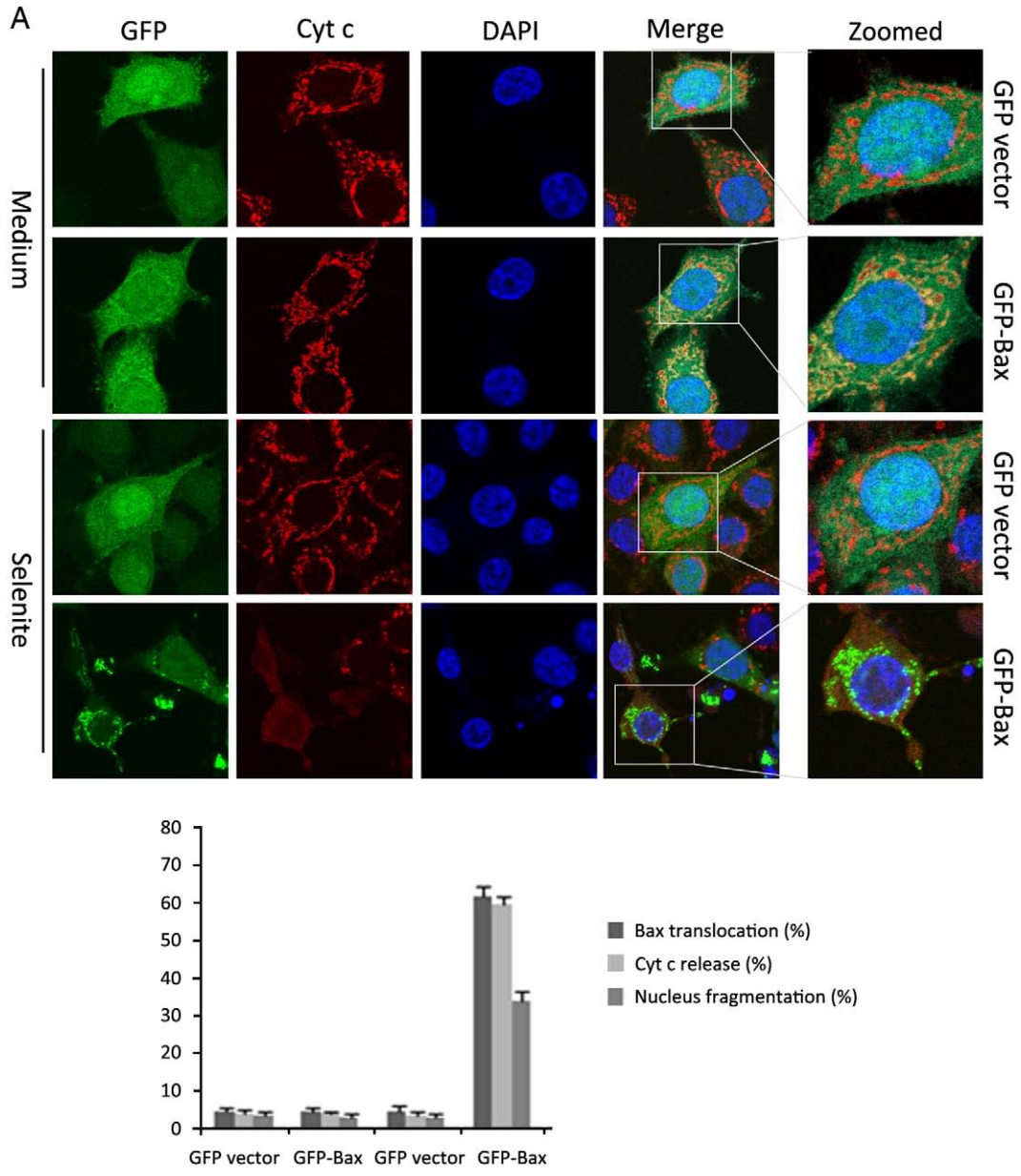

Fig. 2. Selenite induces Bax translocation, conformational change, and oligomerization in a Bcl-xL-inhibitable manner. (A) Confocal fluorescence microscopy demonstrating the distribution of GFP–Bax and cytochrome *c;* original magnification, 63× . SW480 cells were stably transfected with GFP vector and GFP–Bax constructs; these cells were treated with 10 μM selenite for 6 h. Cytochrome *c* is immunostained with mouse monoclonal antibody and Texas red-conjugated goat anti-mouse second antibody; the nuclei were stained with DAPI. Results of quantitative analysis are shown in the graph; at least 300 cells were counted in each group. (B) SW480 cells coexpressing GFP–Bax and Bcl-xL or control vector were treated with 10 μM selenite for 6 h and analyzed as above. Results of quantitative analysis and Western blot analysis of Bcl-xL in the Bcl-xL construct and the empty vector transfectants are shown. (C) Bax oligomerization induced by selenite. GFP–Bax transfectants were treated with 10 μM selenite for the indicated durations and then subjected to cross-linking with DSS followed by immunoblot with GFP antibody. DMSO was the solvent control. SW480 cells coexpressing GFP–Bax and Bcl-xL or control vector were treated with selenite for 12 hours, and subjected to crosslinking. (D) Conformational change in Bax detected by immunoprecipitation with 6A7 anti-Bax antibody followed by Western blot with GFP antibody. SW480 cells coexpressing GFP–Bax and Bcl-xL or control vector were treated with 10 μM selenite for the indicated durations, with the whole-cell lysate as loading control.

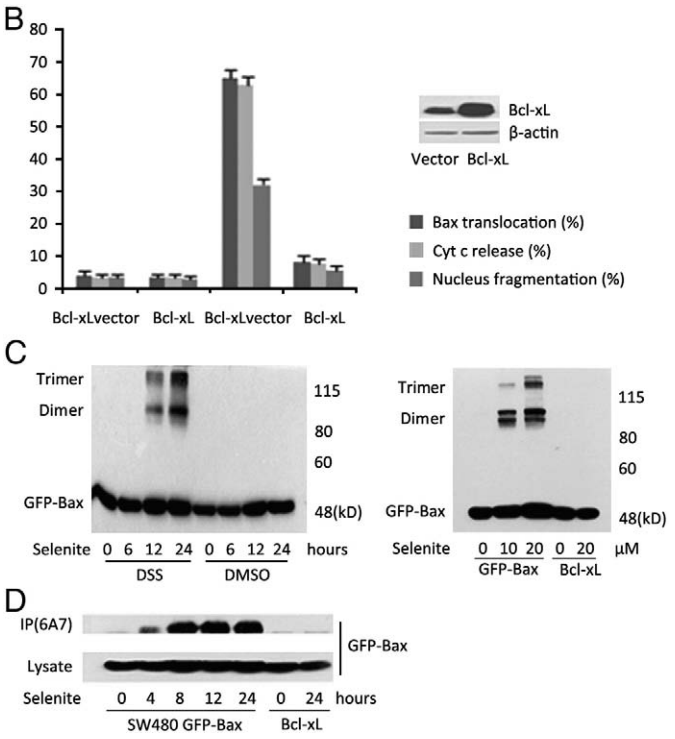

Fig. 2 (*continued*).

constructed. In living cells, GFP and GFP–Bax fusion proteins were diffuse in the cytosol, and cytochrome *c* was localized in mitochondria. After treatment with 10 μM selenite for 6 h, GFP–Bax protein became associated with mitochondria and cytochrome *c* was released into the cytosol in about 60% of cells, whereas no significant changes were observed in cells expressing an empty GFP vector at the same time (Fig. 2A). DAPI staining showed that about 30% of cells displayed nucleus fragmentation (Fig. 2A). These results clearly showed that selenite induces Bax translocation to mediate cytochrome *c* release and apoptosis. Endogenous Bax is not activated at this time point in the absence of exogenous Bax (data not shown), which could sensitize the cells toward apoptosis induced by selenite.

It is postulated that Bax undergoes both an unfolding of the hydrophobic C-terminus from its hydrophobic groove and a change in conformation in the N-terminus of the protein [12]. The 6A7 anti-Bax antibody specifically recognizes the epitope exposed by the conformational change in the N-terminus [18]. Using this antibody, we found that the conformationally changed Bax was detectable in SW480 cells starting from 4 h after treatment with 10 μM selenite (Fig. 2D). Insertion of the α5,6 helix into the membrane and oligomerization are possibly requisite for cytochrome *c* release [19]. We used the membrane-permeative cross-linking agent DSS to detect Bax oligomers, as shown in Fig. 2C. After 12 h of 10 μM selenite treatment, two GFP–Bax-immunoreactive bands of approximately 90 and 140 kDa were detected, which could represent the dimer and trimer of GFP–Bax, respectively. Compared to Bax conformational change and translocation, oligomerization is a late event, which culminated at 12 h after treatment. In addition, selenite induced Bax oligomerization in a dose-dependent manner (Fig. 2C).

A variety of genetic and biochemical studies suggest that antiapoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-xL operate as upstream regulators that oppose the intrinsic death-inducing actions of Bax and Bak at mitochondrial membranes [20,21]. We expressed Bcl-xL in the GFP–Bax-overexpressing SW480 cells and found that Bax translocation, cytochrome *c* release, and nuclear condensation induced by selenite were significantly inhibited (Fig. 2B). Consistently, the conformational change and oligomerization in Bax (Figs. 2C and D) were totally abrogated. These results suggest that Bax is activated as an important effecter mediating cytochrome *c* release induced by selenite in SW480 cells.

Selenite induces Bax-dependent cell death in colon cancer cells

It has been established that cells from Bax/Bak double-knockout mice are resistant to stimuli that are known to kill via the mitochondria-dependent pathway of cell death, including irradiation, DNA-damaging drugs, growth factor deprivation, and agents that induce ER stress [20]. MEF cells from wild-type and $Bax^{-/-}/Bak^{-/-}$ mice were treated with various concentrations of selenite for 36 h, and we consistently found that Bax/Bak-deficient cells were completely resistant to selenite-induced cell death even at 20 μM concentration (Fig. 3A). To further clarify the role of Bax in selenite-induced apoptosis in colon cancer cells, wild-type and Bax-deficient HCT116 cells were treated with selenite for 24 h. We found that Bax-deficient cells were resistant to selenite treatment compared to wild-type HCT116 cells (Fig. 3B). Collectively, these data strongly suggest that selenite kills cells via the Bax-dependent pathway.

Bax activation induced by selenite is redox dependent and is associated with thiol modification

Generation of reactive oxygen species is implicated in the chemopreventive effect of selenite in various cancer models including prostate cancer, hepatoma, and mesothelioma [4,22]. We also observed an increase in intracellular ROS levels in SW480 cells (Fig. 4A) after the treatment with selenite. The SOD mimic MnTMPyP

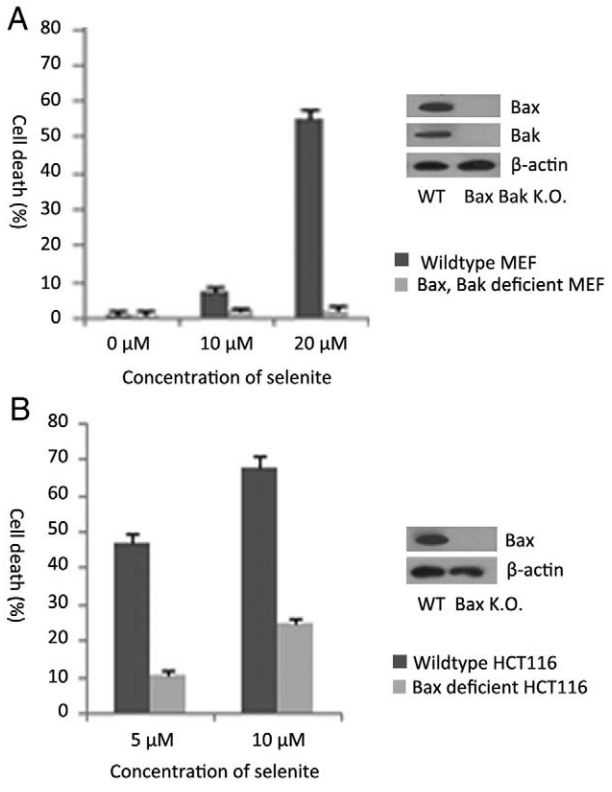

Fig. 3. Selenite induces Bax-dependent cell death in colon cancer cells. (A) Resistance of Bax/Bak knockout cells to selenite-induced cell death. Wild-type and Bax/Bak knockout MEF cells were treated with selenite for 36 h and subjected to trypan blue staining. At least 300 cells were counted in each group. (B) Resistance of Bax knockout HCT116 cells to selenite-induced cell death. Wild-type and Bax knockout HCT116 cells were treated with selenite for 24 h and subjected to trypan blue staining. At least 300 cells were counted in each group.

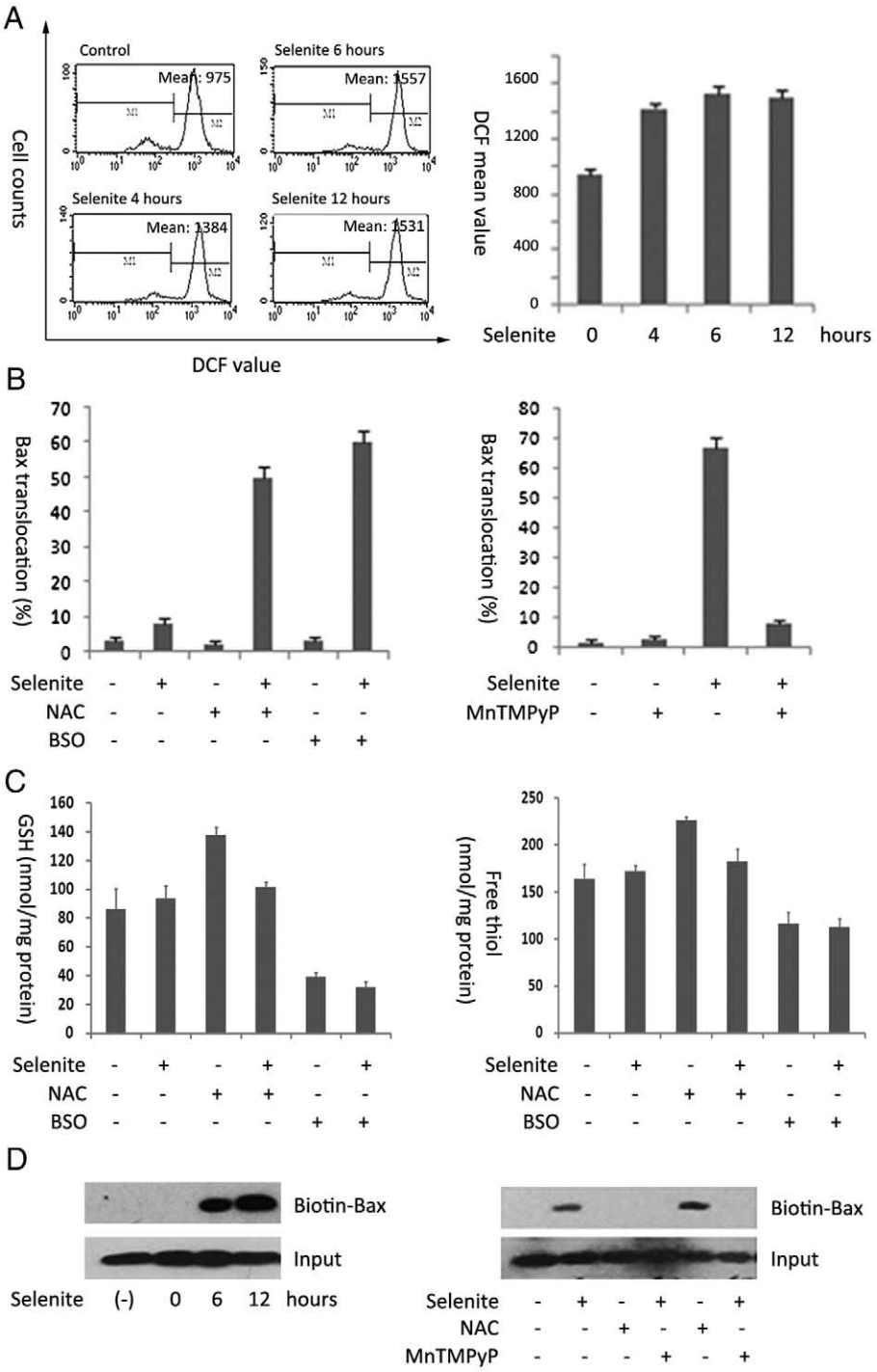

Fig. 4. Bax activation induced by selenite is redox dependent and is associated with thiol modification. (A) Flow-cytometric analysis of cellular ROS level by CM-H_2DCFDA staining. SW480 cells were treated with 10 μM selenite for the indicated durations. (B) Selenite-induced Bax translocation is potentiated by NAC and BSO and eliminated by MnTMPyP. SW480 cells stably expressing GFP–Bax were preincubated with 1 mM NAC or 200 μM BSO for 2 h, washed with DMEM, and then treated with 10 μM selenite for 4 h or were treated with 10 μM selenite combined or not with 5 μM MnTMPyP for 12 h. At least 300 cells were counted in each group. (C) Free thiol and GSH levels in SW480 treated with selenite combined or not with NAC and BSO. (D) Cysteine modification of Bax induced by selenite treatment. SW480 cells were treated with 10 μM selenite for 0, 6, and 12 h, or in combination with NAC and MnTMPyP as above, and then subjected to MPB labeling and purification. (—) is the negative control indicating sufficient blocking of free thiols. All data are representative of three independent experiments.

[5] could strongly inhibit selenite-induced ROS production and subsequent Bax activation in GFP–Bax-overexpressing SW480 cells (Fig. 4B). In addition, we used the compounds NAC and buthionine sulfoximine (BSO), both of which were reported to interfere with intracellular GSH levels and enhance selenite-induced oxidative stress [5,22], and found that both potentiated selenite-induced Bax translocation (Fig. 4B). We did not observe any global changes in GSH levels or free thiol levels when the cells were treated with 10 μM selenite (Fig. 4C), despite the occurrence of changes when cells were treated with 20 μM selenite (Fig. S2). We suggest that a unique distribution pattern of GSH/free thiol may exist within the cells. The environment in the proximity of mitochondria is more oxidized owing to the free radicals released from mitochondria, thus promoting Bax oxidation and translocation. These results clearly suggest that

selenite-induced Bax activation is closely related to perturbation of the intracellular redox homeostasis.

We then asked if Bax is chemically modified after treatment with selenite. Bax has two highly conserved cysteine residues, and Ganther and colleagues proposed that thiol modification induced by selenium compounds might occur via S–Se or S–S bonds [23]. We thus used biotin-conjugated thiol-reactive probes to detect selenium-induced protein modification following the method used by Toledano and colleagues [24]. In wild-type SW480 cells, we found that cysteines in endogenous Bax were modified by a DTT-reducible covalent bond after selenite treatment, in a time-dependent manner (Fig. 4D). NAC treatment slightly increased the extent of Bax modification, whereas MnTMPyP, which inhibits intracellular ROS generation, completely abolished this modification induced by selenite (Fig. 4D). These data suggest that cysteine residues of Bax could be modified by intracellular ROS after selenite treatment.

Cys 62 and Cys 126 of Bax are necessary for Bax activation induced by selenite

To further substantiate the possibility that ROS induced by selenium metabolites could directly react with the highly conserved Cys residues of Bax at positions 62 and 126, SW480 cell clones stably expressing the GFP-Bax (wt) plasmid and recombinant GFP–Bax plasmids carrying mutations corresponding to both cysteines, GFP-Bax (C62S/C126S), were generated. After treatment with 10 μM selenite for 6 hours, GFP-Bax (wt) protein became associated with mitochondria and cytochrome *c* was released into the cytosol in about 60% cells expressing GFP-Bax (wt) protein, whereas GFP-Bax (C62S/

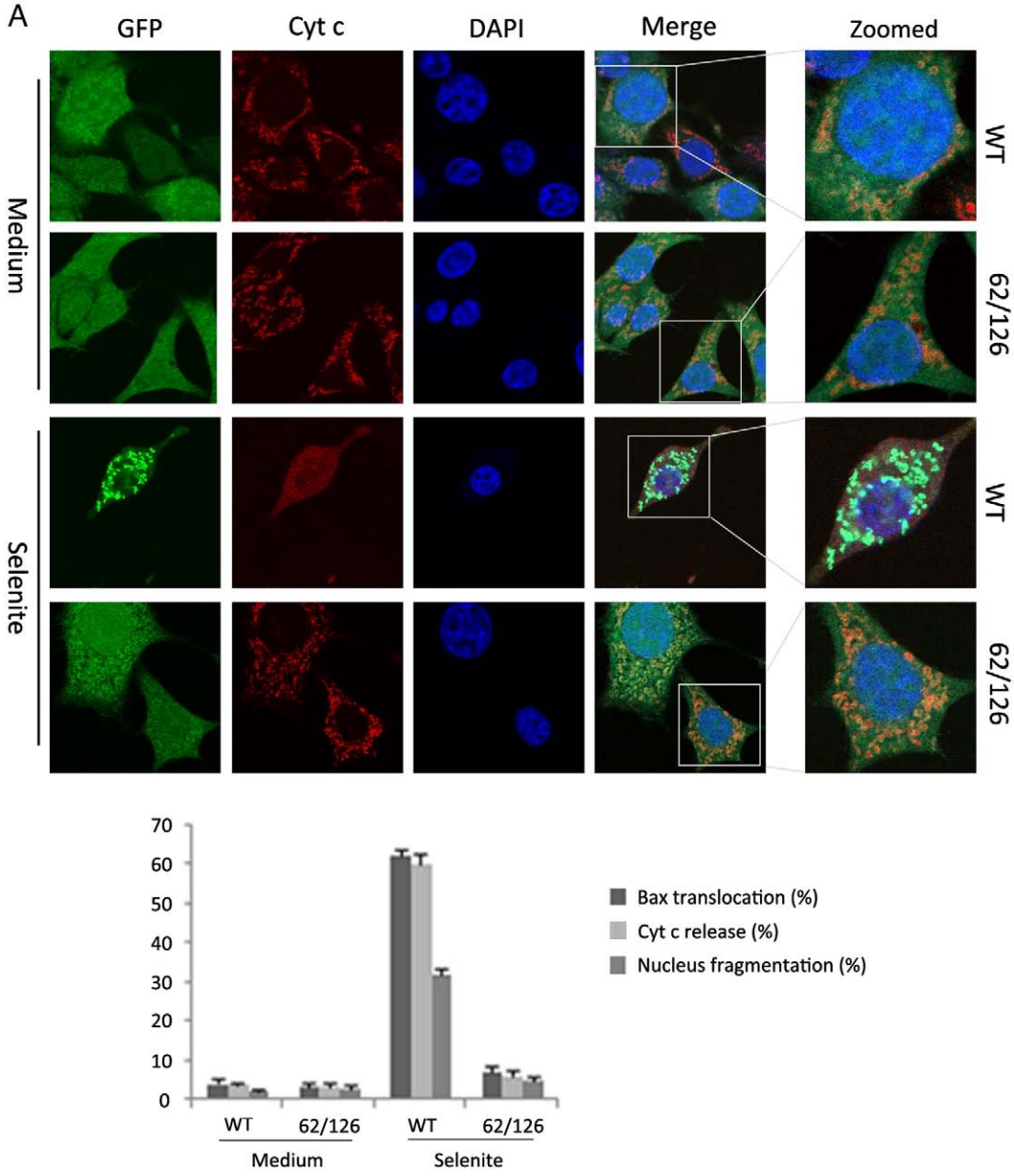

Fig. 5. Cys 62/126 of Bax are necessary for Bax activation induced by selenite. (A) Confocal fluorescence microscopy demonstrating the distribution of GFP–Bax and cytochrome *c*, original magnification, 63×. SW480 cells were stably transfected with wild-type GFP–Bax and C62S/C126S GFP–Bax constructs. These cells were treated with 10 μM selenite for 6 h. (B) Quantitative analysis results demonstrating effects of H_2O_2 on Bax translocation and cell apoptosis in SW480 cells expressing wild-type GFP–Bax and C62S/C126S GFP–Bax. Cells were treated with 25 μM H_2O_2 for 6 h. (C, D) Distinct effects of selenite on Bax conformational change and oligomerization in SW480 cells expressing wild-type GFP–Bax and C62S/C126S GFP–Bax. Cells were treated with 10 μM selenite for 4 and 12 h, respectively. (+) is a positive control in which cells were lyzed with NP40 and Bax underwent conformational change necessarily. (E) Effects of camptothecin and etoposide in SW480 cells expressing wild-type GFP–Bax and C62S/C126S GFP–Bax. Cells were treated with 1 μM camptothecin and 10 μM etoposide for 12 h. All data are representative of three independent experiments.

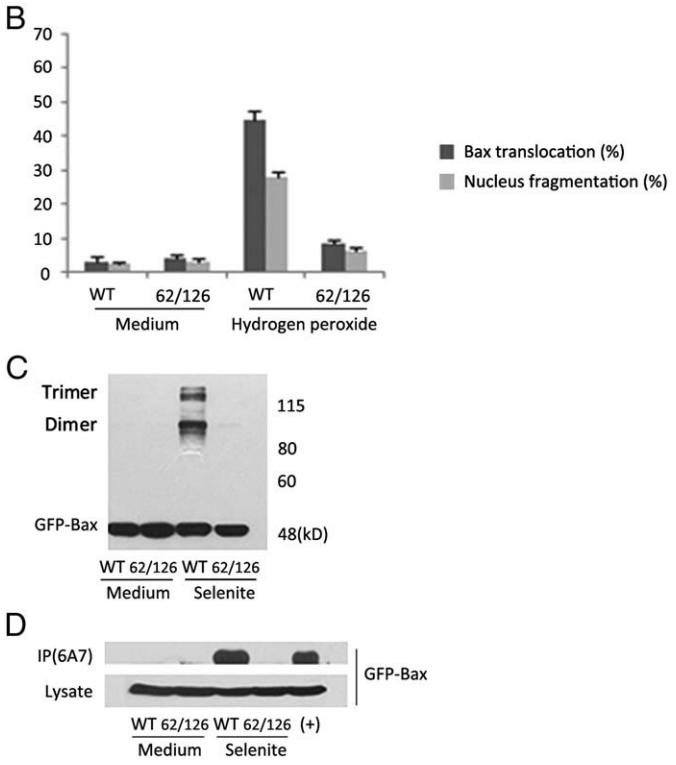

Fig. 5 (*continued*).

126S) protein was still diffuse in the cytosol and cytochrome *c* was localized in mitochondria (Fig. 5A). We then used the same method to detect Bax conformational change and oligomerization and found that conformationally changed GFP-Bax (wt) was detectable from 4 h and oligomerized GFP-Bax (wt) was detectable 12 h after 10 μM selenite treatment. However, no conformationally changed and oligomerized GFP-Bax (C62S/C126S) was detected after the same time (Figs. 5C and D). We also used H_2O_2 in the same system and demonstrated that SW480 cells expressing GFP-Bax (C62S/C126S) were also resistant to ROS-induced activation compared to cells expressing GFP-Bax (wt) (Fig. 5B). As a control, we used the DNA-damaging drugs etoposide and camptothecin and found that they induced no discrimination in Bax activation in SW480 cells expressing GFP-Bax (wt) and GFP-Bax (C62S/C126S) (Fig. 5E). Taken together, the results clearly show that modification of Cys 62/126 in Bax might be important for Bax conformational change and subsequent activation induced by selenite.

Selenite attenuates the growth of colon cancer cells in nude mice

We next tested the effects of selenite on the growth of colon cancer cells in vivo. SW480 colon cancer cells were implanted in immunodeficient nude mice. Seven days after implantation, when most of the tumors were palpable, mice underwent intraperitoneal treatment with selenite (2 mg/kg/day). The average tumor volume of the treated group was significantly reduced compared to that of the control group (Fig. 6A), and this dose of selenite inhibited tumor growth in the mice without any adverse effects on body weight and activity (Fig. 6B). Moreover, in situ detection of apoptotic cells by TUNEL assay in tumors from both groups showed an increase in apoptotic cells after selenite treatment (Fig. 6C).

Discussion

The principal finding in this paper concerns the molecular details of how Bax is activated and translocated to the mitochondria by selenite. Selenite induces the oxidative stress response and an increase in intracellular ROS levels (Fig. 4A), which causes Bax activation and its subsequent translocation onto mitochondria. We further identified the critical role of the conserved Cys 62/126 residues of Bax in its activation during selenite-induced apoptosis. Mutation of Cys 62/126 abolished the proapoptotic activity of Bax induced by selenite but not by DNA-damage inducers. Moreover, our data validate Bax as an important target for selenite in colon cancer cells.

Previous reports have documented that selenite-induced intracellular free radical generation is related to subsequent apoptosis [4,25]. However, how these events are coupled with the mitochondrial apoptotic machinery remains elusive. It is reported that selenium compounds induce mitochondrial permeability transition [26]; Li and colleagues demonstrated that ROS could up-regulate Bim, which is responsible for Bax activation [27]. Cysteine residues can be oxidized by intracellular H_2O_2 to form a Cys–SOH intermediate, which then reacts with a neighboring cysteine to form a disulfide [28]. D'Alessio and colleagues showed that disulfide bonds formed between two Bax molecules play an important role in Bax translocation to mitochondria [29]. We detected thiol modification on Bax in response to selenite. Furthermore, selenite-induced Bax modification was strongly inhibited by an SOD mimic and potentiated by NAC and BSO (Fig. 4B). As a reservoir of free thiols, glutathione scavenges ROS generated by selenite, but also reacts with selenite to produce more ROS [22]. This could explain our data showing that Bax translocation was potentiated in both cases (Fig. 4B). Mutation of the cysteines in positions 62/126 abolished the Bax conformational change and translocation in response to selenite, but not DNA-damaging compounds (Fig. 4). These data suggest that mutation alone does not alter Bax conformation and that DNA-damaging agents could activate JNK, which phosphorylates Bax for its translocation onto mitochondria [30]. Thus, it is likely that intracellular ROS generated from mitochondria by selenite function as a redox signal and result in exposure of the BH3 domain and C-terminal transmembrane domain, leading to its lethal attack on mitochondria. These data are consistent with our previous observations showing that intracellular ROS could directly activate Bax by reacting with its Cys residues for subsequent apoptosis [15]. It is known that selenite is a direct substrate for thioredoxin reductase (TrxR) from mammalian cells. In the presence of oxygen, the metabolic product of selenite, i.e., selenide, will also undergo a redox cycle with thiols (R–SH) to generate massive amounts of ROS and increase oxidative stress, which was explored for cancer therapy [25] because up-regulation of TrxR was found in a number of cancers [31]. Indeed, we observed that selenite inhibits TrxR (data not shown), which may be causally linked with an increase in ROS levels and Bax activation in SW480 cells. We also recently showed that Cys modification of thioredoxin by ROS could reverse its protective function and lead to subsequent apoptosis [9].

There is a growing body of evidence showing the differences in sensitivity between tumor cell lines and normal cells in response to selenite, including prostate cancer [32] and malignant mesothelioma [4]. In our experiments, we found that SW480 cells were much more sensitive to selenite than their normal counterparts (Fig. 1D). In addition, wild-type MEF cells are resistant to selenite-induced cell death at 10 μM. In supporting the notion of using selenite in cancer therapy, we have shown that a supranutritional dose of selenite could significantly inhibit xenograft tumor growth in nude mice without obvious side effects. Protein thiol modification of apoptosis-related proteins has been proposed as an explanation for the chemotherapeutic effects of selenium. Several proteins, including the transcription factor NF-κB [33], signaling module PKC [34], and mitochondrial membrane protein VDAC [35], were evidenced as targets for direct thiol modification by selenium. Our results suggest that sensing the changes in intracellular redox status directly by Bax or other proapoptotic Bcl-2 family proteins

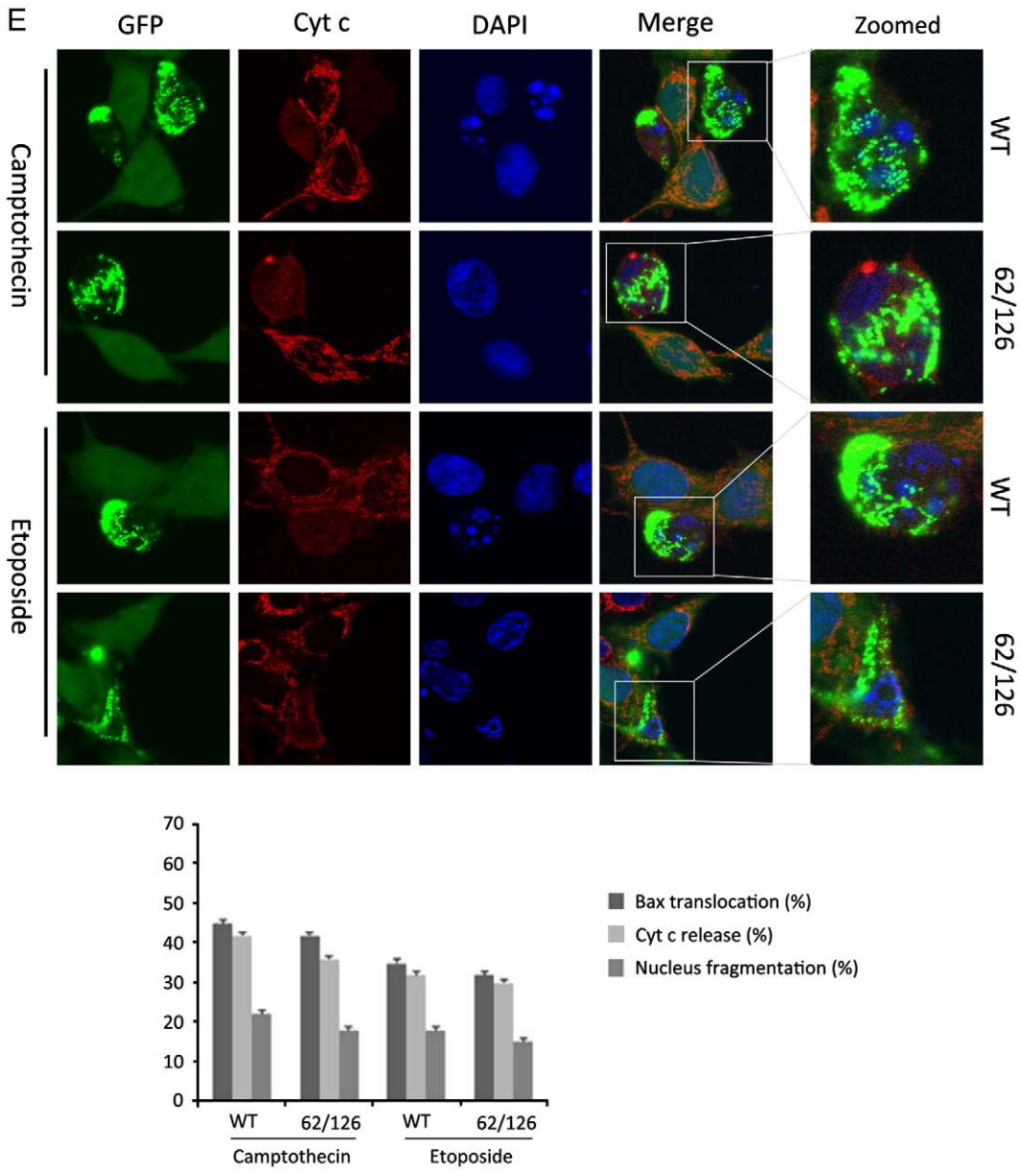

Fig. 5 (*continued*).

might provide advantages to killing tumor cells with selenite, for it could circumvent the upstream inhibitory signals that are usually up-regulated in tumor cells. Recently, several reports have demonstrated the advantages of using selenium in combination with chemotherapeutic drugs such as doxorubicin [27] and TRAIL [36]. Activation of Bax might be a potential mechanism responsible for the synergic effects. Overall, our results support the notion that apoptosis is an important mechanism for the anti-cancer effects of selenium compound [5]. We suggest that using Bax as a target for thiol modification induced by selenite might be beneficial for eliminating cancer cells and attenuating carcinogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2009.01.026.

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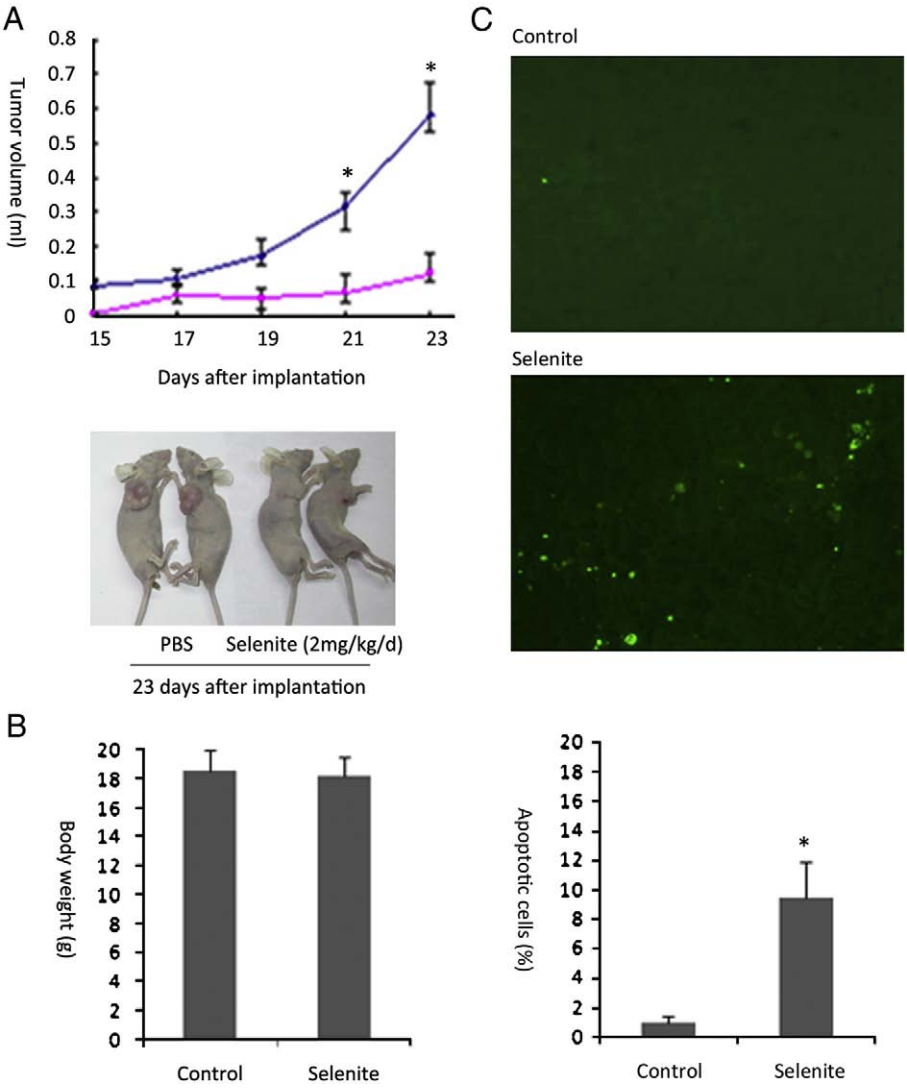

Fig. 6. Selenite attenuates the growth of colon cancer cells in nude mice. (A) Tumor volume curves and representative images of mice bearing xenograft tumors formed by injection of SW480 cells treated with PBS or selenite (2 mg/kg/day). *$p<0.05$. (B) Average body weight of mice at 23 days after implantation. There is no statistical significance between the control and the selenium groups. (C) Representative images and quantitative analysis of in situ labeling of apoptotic cells using the TUNEL assay, original magnification, 10×. *$p<0.05$.

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