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Original Contribution

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

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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.

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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