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Sulforaphane induces reactive oxygen species-mediated mitotic arrest and subsequent apoptosis in human bladder cancer 5637 cells

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ABSTRACT

The present study was undertaken to determine whether sulforaphane-derived reactive oxygen species (ROS) might cause growth arrest and apoptosis in human bladder cancer 5637 cells. Our results show that the reduced viability of 5637 cells by sulforaphane is due to mitotic arrest, but not the G2 phase. The sulforaphane-induced mitotic arrest correlated with an induction of cyclin B1 and phosphorylation of Cdk1, as well as a concomitant increased complex between cyclin B1 and Cdk1. Sulforaphane-induced apoptosis was associated with the activation of caspase-8 and -9, the initiators caspases of the extrinsic and intrinsic apoptotic pathways, respectively, and activation of effector caspase-3 and cleavage of poly (ADP-ribose) polymerase. However, blockage of caspase activation inhibited apoptosis and abrogated growth inhibition in sulforaphane-treated 5637 cells. This study further investigated the roles of ROS with respect to mitotic arrest and the apoptotic effect of sulforaphane, and the maximum level of ROS accumulation was observed 3 h after sulforaphane treatment. However, a ROS scavenger, *N*-acetyl-L-cysteine, notably attenuated sulforaphane-mediated apoptosis as well as mitotic arrest. Overall, these results suggest that sulforaphane induces mitotic arrest and apoptosis of 5637 cells via a ROS-dependent pathway.

1. Introduction

Bladder cancer is the fourth commonest male and ninth commonest female malignant disease in the United States (Abdollah et al., 2013), although the incidence of bladder cancer in Asia is much lower (Cheon et al., 2002; Kakehi et al., 2010). Due to its expanded therapy and the need for lifelong surveillance, bladder cancer is expensive to treat. Despite recent advances in surgical and chemotherapeutic procedures, the 5-year survival rate in patients with invasive and metastatic bladder cancer remains very low (Rosenberg et al., 2005). It is therefore important to develop other effective strategies to improve the survival rate for bladder cancer patients. In light of this, studies on promising dietary components may offer a new strategy for improvement of bladder cancer prognosis.

Recent epidemiologic data indicate that populations with a high dietary intake of cruciferous vegetables have lower incidences of bladder cancer (Liu et al., 2013; Tang et al., 2010; Michaud et al., 1999), which is attributed, in part, to the high content of glucosinolates in many cruciferous vegetables, including broccoli (Tang and Zhang, 2004). Glucosinolates are converted to isothiocyanates by myrosinase, an enzyme which is released when the cell structure of plants has been disrupted through chewing, chopping, or digestion (Shapiro et al., 2001). As a major group of active phytochemicals in cruciferous vegetables, isothiocyanates have shown anticancer activity through the inhibition of carcinogen-activating enzymes, as well as through the induction of carcinogen-detoxifying enzymes, differentiation, cell cycle arrest and apoptosis (Hanlon et al., 2009; Munday et al., 2006). Among isothiocyanates, sulforaphane [1-isothiocyanato-4-(methylsulfinyl)butane] is abundant in broccoli and has been shown to exhibit anti-cancer activities in a

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wide variety of tumors by inducing cell cycle arrest and apoptosis, and by inhibiting metastasis (Cheung and Kong, 2010; Clarke et al., 2008; Gamet-Payrastre, 2006; Lee et al., 2013). This compound has also shown anti-bladder cancer activity *in vitro* and *in vivo*; the reported mechanisms of which include modulation of cyclooxygenase-2 expression associated with p38 mitogen activated protein kinase activation in T24 bladder cancer cells (Shan et al., 2010), inhibition of 4-aminobiphenyl-induced DNA damage in RT4 bladder cancer cells and in mouse bladder tissue (Ding et al., 2010), and inhibition of invasion and metastasis by suppressing epithelial-to-mesenchymal transition process (Shan et al., 2013).

Many cytotoxic agents and/or DNA damaging agents arrest cell cycling at G1, S or G2/M phase, inducing apoptotic cell death (Naithani et al., 2008; Canavese et al., 2012). Cell cycle checkpoints may function to ensure cells to have time for DNA repair (Schwartz and Shah, 2005; Dean et al., 2012). Therefore, recent studies have offered novel insights into the molecular mechanisms of sulforaphane-induced cell cycle arrest and apoptosis. It has been determined in previous studies that sulforaphane treatment generates reactive oxygen species (ROS) to trigger signal transduction culminating in cell cycle arrest at G2/M phase and/or apoptosis in multiple cancer types (Lee et al., 2012; Singh et al., 2004, 2005; Mi et al., 2011; Cho et al., 2005). We also previously found that cellular ROS generation by sulforaphane plays a pivotal role in the initiation of sulforaphane-triggered apoptotic death in human hepatocellular carcinoma and leukemia U937 cells (Moon et al., 2010; Choi et al., 2008). However, the ROS generation by sulforaphane is tumor cell specific, since normal cell lines were resistant to cell cycle arrest and apoptosis by sulforaphane (Meeran et al., 2010; Kallifatidis et al., 2009; Antosiewicz et al., 2008). Meanwhile, Shan et al. (2006) reported that sulforaphane-induced apoptosis of bladder cancer T24 cells is related to the induction of G1 phase arrest of cell cycle, through up-regulation of cyclin-dependent kinase (Cdk) inhibitor p27 expression. However, sulforaphane treatment leads to accumulation in the G2/M phase of the cell cycle and then apoptotic cell death in other bladder cancer cell lines (RT4, J82 and UMUC3) (Abbaoui et al., 2012). These studies cumulatively indicate that sulforaphane may affect different signaling pathways depending on the cell type or culture conditions used. Nevertheless, the roles of ROS-mediated induction of cell cycle arrest and apoptosis by sulforaphane in human bladder cancer cells has still not been elucidated.

Thus, in the present study, the molecular mechanisms of ROS responsible for anti-cancer regulation of sulforaphane were examined in human bladder cancer 5637 cells. Our data demonstrate that sulforaphane induces mitotic arrest, but not G2 phase, and also induces caspase-dependent apoptosis by the accumulation ROS, thereby representing sulforaphane as a promising therapeutic agent for the treatment of bladder cancer.

2. Materials and methods

2.1. Reagents

Sulforaphane was purchased from Sigma–Aldrich Chemical Co. (St. Paul, MN) and dissolved in dimethyl sulfoxide (DMSO, Sigma–Aldrich), and then diluted with the medium to the desired concentration prior to use. RPMI-1640 medium and fetal bovine serum (FBS) was obtained from GIBCO-BRL (Gaithersburg, MD). Caspase activity assay kits and enhanced chemiluminescence (ECL) kit were purchased from R&D Systems (Minneapolis, MN) and Amersham (Arlington Heights, IL), respectively. 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT), 4,6-diamidino-2-phenyllindile (DAPI), propidium iodide (PI), 5,5′, 6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1) and N-acetyl L-cysteine (NAC) were obtained from Sigma–Aldrich. Pan-caspase inhibitor, z-VAD-fmk, was obtained from Calbiochem (San Diego, CA). 2′,7′-dichlorofluorescein diacetate (DCFDA) and fluorescein-isothiocyanate (FITC)-Annexin V were purchased from Molecular Probes (Eugene, OR) and PharMingen (SinDiego, CA), respectively. Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Chemicon (Temecula, CA) and Sigma–Aldrich. Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin were purchased from Amersham (Arlington Heights, IL). All other chemicals were purchased from Sigma–Aldrich.

2.2. Cell culture and cytotoxicity assay

5637 Cells were purchased from American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine and penicillin/streptomycin. In order to measure the inhibition of 5637 cell proliferation by sulforaphane, cells were plated in 6-well culture plates (1×10^5 cells/well) and allowed to adhere overnight, and then treated with different concentrations of sulforaphane for 48 h. After treatment, 0.5 mg/ml MTT solution was added, and the plates were incubated for an additional 3 h at 37 °C. The medium was subsequently removed, and dimethyl sulfoxide (Sigma–Aldrich) was added. Optical density was measured at 540 nm using a microplate spectrophotometer (Dynatech Laboratories, Chantilly, VA).

2.3. Morphological observation of nuclear change

After culture with various concentrations of sulforaphane, cells were washed with phosphate-buffered saline (PBS) and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were washed with PBS, and stained with 2.5 μg/ml DAPI solution for 10 min at room temperature. The cells were analyzed using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

2.4. Cell cycle analysis

Following treatment with sulforaphane, cells were trypsinized, washed with PBS, and fixed in 75% ethanol at 4 °C for 30 min. The cells were suspended in cold 50 μ/ml PI solution and 0.1% Triton X-100 (Sigma–Aldrich), and incubated at room temperature in the dark for 30 min. A FACScan flow cytometry system (Becton Dickinson, San Jose, CA) was used for performance of flow cytometry analysis.

2.5. Annexin-V staining

To analyze apoptosis, cells were treated with the indicated concentrations of sulforaphane for 48 h, and apoptosis was analyzed by staining phosphatidylserine translocation with 5 μM FITC-annexin V for 30 min at room temperature in the dark according to the manufacturer's instructions. Flow cytometric analysis was performed as described previously (Li and Gao, 2013).

2.6. Total protein extraction, immunoprecipitation and Western blot assay

For isolation of total protein fractions, cells were collected, washed twice with cold PBS, and lysed with cell lysis buffer [20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 0.5 g/ml leupeptin, 1% Na_3VO_4, 1 mM phenylmethane-sulfonyl fluoride]. For Western blot assay, the total proteins were separated by SDS–polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) by electroblotting. After being blocked with blocking solution (1% BSA in PBS plus 0.05% Tween-20) at room temperature for 1 h, each membrane was incubated for 2 h at room temperature or overnight at 4 °C with primary antibodies, and then the membranes were probed for 1 h at room temperature with the peroxidase-labeled secondary antibodies. Detection was performed by the ECL Western blotting detection kit according to the manufacturer' instructions. In a parallel experiment, interaction of cyclin B1 with Cdk1 was analyzed by immunoprecipitation and subsequent Western blot analysis. For this study, lysates representing 500 μg of protein were precleared by the addition of protein A-Sepharose beads (Sigma–Aldrich), followed by rocking at 4 °C for 1 h. To the precleared lysate, 2 μg of ant-Cdk1 antibody was added and then rocked at 4 °C for 4 h. The antibody-associated complexes were collected by adding protein A-Sepharose beads p and incubating for a further 2 h at 4 °C with constant rotation. The immunoprecipitates were washed three times with lysis buffer and, after removal of as much liquid as possible, were resuspended in SDS sample buffer for Western blot analysis using anti-cyclin B1 and Cdk1 antibodies.

2.7. Analysis of caspase-3, -8 and -9 activities

To evaluate caspase activity, cell lysates were prepared after treatment with sulforaphane. Assays were performed in 96-well plates by incubating 20 μg cell lysates in 100 mL reaction buffer containing 5 μM of colorimetric tetrapeptides [Asp-Glu-Val-Asp (DEAD) for caspase-3; Ile-Glu-Thr-Asp (IETD) for caspase-8; Leu-Glu-His-Asp (LEHD) for caspase-9] labeled with p-nitroaniline (pNA) at 37 °C for 2 h according to the manufacturer's protocol. Thereafter, the optical density of the reaction mixture was measured spectrophotometrically at a wavelength of 405 nm using a microplate spectrophotometer.