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Original Contribution

Thymoquinone suppresses growth and induces apoptosis via generation of reactive oxygen species in primary effusion lymphoma

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ABSTRACT

We provide evidence that thymoquinone (TQ), a natural compound isolated from *Nigella sativa,* induces growth inhibition and apoptosis in several primary effusion lymphoma (PEL) cell lines. Our data demonstrate that TQ treatment results in down-regulation of constitutive activation of AKT via generation of reactive oxygen species (ROS) and it causes conformational changes in Bax protein, leading to loss of mitochondrial membrane potential and release of cytochrome *c* to the cytosol. This leads to activation of caspase-9, caspase-3, and polyadenosine 5′-diphosphate ribose polymerase cleavage, leading to caspase-dependent apoptosis. Pretreatment of PEL cells with *N*-acetylcysteine, a scavenger of ROS, prevented TQ-mediated effects. In addition, subtoxic doses of TQ sensitized PEL cells to TRAIL via up-regulation of DR5. Altogether, these findings demonstrate that TQ is a potent inducer of apoptosis in PEL cells via release of ROS. They also raise the possibility that incorporation of TQ in treatment regimens for primary effusion lymphomas may provide a novel approach to sensitizing malignant cells and provide a molecular basis for such future translational efforts.

Primary effusion lymphoma (PEL) is a subtype of non-Hodgkin B cell lymphoma that mainly presents in patients with advanced AIDS, but is sometimes also found in human immunodeficiency virus-negative individuals [1,2]. PEL cells grow as a lymphomatous effusion in body cavities and are infected with Kaposi sarcoma-associated herpesvirus (KSHV/HHV8). Most cases show dual infection with Epstein–Barr virus (HHV4) [3]. Pleural and abdominal effusions from patients with AIDS PEL contain a number of cytokines, which serve as autocrine growth factors [4,5]. For example, IL-10 has been reported to serve as an autocrine growth factor for AIDS-related B cell lymphoma [6]. Recently, it has also been shown that PEL cells use viral IL-6 and IL-10 in an autocrine fashion for their survival and proliferation [6].

A number of constitutively activated signaling pathways play critical roles in the survival and growth of PEL cells. These include JAK/STAT, NF-κB, and PI3-kinase [7–10]. The KSHV protein K1 has been shown to regulate a number of survival proteins. Studies have shown that transgenic mice with K1 showed constitutive activation of NF-κB and Oct-2 as well as activation of Src-kinase Lyn [11]. Furthermore, K1 protein activates the PI3-kinase/AKT pathway in B lymphocytes and protects cells from FKHR- and FAS-mediated apoptosis [12]. A study by Cannon et al. also demonstrated that vGPCR induces activation of the transcriptional factors AP1 and CREB in PEL cells [13].

Thymoquinone (TQ) is a bioactive compound isolated from *Nigella sativa* Linn [14]. Extracts prepared from *N. sativa,* known as black seed, have been used for medical purposes for centuries for a number of diseases [14,15]. TQ is the active ingredient of black seed and has been shown to possess antitumor activities against a broad spectrum of cancer cells, including colon, ovarian, lung, osteosarcoma, and myeloblastic leukemia [16–20]. TQ has also been shown to inhibit chemically induced carcinogenesis in mice [21,22]. More recently, it was demonstrated that TQ augments the antitumor activity of standard cancer chemotherapeutic agents in pancreatic cancer cells in vitro and in vivo [23].

In this study, we investigated the antitumor activity of TQ against human primary effusion lymphoma cell lines. Our data provide the first evidence that TQ induces apoptosis of PEL cells via a mechanism involving generation of reactive oxygen species (ROS). We also examined the effects of TQ on extrinsic and intrinsic apoptotic pathways. Finally, we studied the effects of TQ in combination with TRAIL on PEL cell lines. Altogether, our data establish TQ as a potent inducer of apoptosis in PEL cells and identify the mechanisms by which such apoptosis is induced.

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Materials and methods

Cell culture

The human PEL cell lines BC-1, BC-3, BCBL-1, and HBL-6 were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin at 37 °C in an humidified atmosphere containing 5% CO_2. All the experiments were performed in RPMI 1640 containing 5% serum.

Reagents and antibodies

Thymoquinone, *N*-acetylcysteine (NAC), and Bax 6A7 monoclonal antibody were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Caspase-9 antibody and zVAD-fmk were purchased from Calbiochem (San Diego, CA, USA). Antibodies against phosphorylated (p) AKT, AKT, p-Bad, and cleaved caspase-3 were purchased from Cell Signaling Technologies (Beverly, MA, USA). Cytochrome *c*, β-actin, caspase-3, and polyadenosine 5′-diphosphate ribose polymerase (PARP) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DR5 antibody was purchased from Cayman Laboratories (Ann Arbor, MI, USA). TUNEL assay kit was obtained from MBL (Watertown, MA, USA). Annexin V was purchased from Molecular Probes (Eugene, OR, USA). Apoptotic DNA ladder kit was obtained from Roche (Penzberg, Germany).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays

The antiproliferative effects of TQ against various PEL cell lines were determined by the MTT dye-uptake method as described earlier [24]. Briefly, 10^4 cells were incubated in triplicate in a 96-well plate in the presence or absence of the indicated test doses of TQ in a final volume of 0.20 ml for 24 h at 37 °C. Thereafter, 25 μl MTT solution (5 mg/ml in water) was added to each well. After 24 h incubation at 37 °C, 0.1 ml extraction buffer (20% SDS) was added, incubation was continued overnight at 37 °C, and then the optical density (OD) at 590 nm was measured. Cell viability was calculated as OD of the experiment samples/OD of the control (untreated) × 100. Replicates of 6 wells for each dosage including vehicle control were analyzed for each experiment.

Cell cycle analysis

Cell lines were treated with or without TQ for 24 h and the cells were washed once with phosphate-buffered saline (PBS) and resuspended in 500 μl hypotonic staining buffer (sodium citrate 250 mg, Triton X-100 0.75 ml, propidium iodide 25 μg, ribonuclease A 5 μg, and water 250 ml) and analyzed by flow cytometry as described previously [25].

TUNEL assay

Cell lines were treated with TQ as described in the legends. Apoptotic cells were measured using the TUNEL assay as described earlier [26]. Briefly, after 24 h of treatment with various concentrations of TQ, 1×10^6 cells were washed twice with PBS containing 0.2% bovine serum albumin (BSA) and fixed with 4% paraformaldehyde at 4 °C for 30 min. This was followed by two washes with PBS containing 0.2% BSA and the cells were permeabilized in 70% ethanol at −20 °C for 30 min. The cells were then washed twice and incubated with 30 μl TdT buffer (TdT buffer II, FITC–dUTP, and TdT in the ratio of 18:1:1) for 1 h at 37 °C. This was followed by two washes with PBS and the cells were resuspended in 500 μl of PBS. Stained cells were analyzed using FACScan flow cytometry equipped with a CellQuest data analysis program (Beckon–Dickinson, San Diego, CA, USA).

Annexin V staining

PEL cell lines were treated with various concentrations of TQ as described in the legends. Cells were harvested and the percentage apoptosis was measured by flow cytometry after staining with fluorescein-conjugated annexin V and propidium iodide (PI; Molecular Probes) as described previously [27]. We scored viable cells as those that are negative for annexin V and PI. Percentage of apoptosis was calculated from the reduction in the number of viable cells between the treated and the untreated samples. The amount of necrotic cells (annexin V negative, PI positive) was always minimal.

Measurement of ROS

ROS production was detected using dihydroethidium and high-performance liquid chromatography (HPLC) as described earlier [28,29]. Briefly, exponentially growing cells were treated with TQ alone or in the presence of 10 mM NAC for the indicated time periods. After treatment, the cells were washed three times with chilled Krebs–Hepes buffer. The cells were then exposed to 25 μM dihydroethidium dissolved in Krebs–Hepes buffer containing 0.1% dimethyl sulfoxide for 20 min at 37 °C. The cells were washed with Krebs–Hepes buffer and incubated for 1 h in Krebs–Hepes buffer at 37 °C. The cells were then spun at 1000 rpm for 10 min and resuspended in ice-cold methanol, homogenized, and filtered through a 0.22-μm filter. Separation of ethidium, oxyethidium, and dihydroethidium was performed using an Atlantis dC18 (4.6 × 150 mm, 5 μm) column equipped with a Waters Alliance 2695 separations module (Waters Associates, Milford, MA, USA).

Soft-agar colony assays

Soft-agar colony experiments were performed according to the manufacturer's protocol (Chemicon International, Temecula, CA, USA). Briefly, after treatment with TQ in the presence or absence of NAC, 2500 cells were plated in 0.5 ml culture medium containing 0.4% (v/v) top agar and 20% FBS layered over a basal layer of 0.8% (v/v) agar and 20% FBS in culture medium and allowed to grow for 4 weeks. After 4 weeks incubation, the cells were stained at a final concentration of 1 mg/ml with cell staining solution that was supplied with the kit.

Cell lysis and immunoblotting

Cells were treated with TQ as described in the legends and lysed as previously described [30]. Briefly, cell pellets were resuspended in phosphorylation lysis buffer (0.5–1.0% Triton X-100, 150 mM NaCl, 1 mM EDTA, 200 μM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1.5 mM magnesium chloride, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin). Protein concentrations were assessed by Bradford assay before the samples were loaded. Equal amounts of proteins were separated by SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore, Billerica, MA, USA). Immunoblotting was performed with various antibodies and visualized using an enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA) method.

Detection of Bax conformational changes

This assay was performed as described previously [31]. Briefly, after treatment with the indicated reagents for the indicated times, cells were harvested and washed with PBS, after which they were lysed with Chaps lysis buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 1% Chaps) containing protease inhibitors as described. Protein concentrations were assessed by Bradford assay and 500 μg of total protein was incubated with 2 μg of anti-Bax 6A7 monoclonal antibody for 2 h at 4 °C. After incubation, 25 μl of protein G beads was added into the reaction and incubated at 4 °C overnight on a shaker with gentle agitation. After washes in lysis buffer,