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The effects of 1,4-benzoquinone on c-Myb and topoisomerase II in K-562 cells☆

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ABSTRACT

Exposure to benzene, a ubiquitous environmental pollutant, has been linked to leukemia, although the mechanism of benzene-initiated leukemogenesis remains unclear. Benzene can be bioactivated to toxic metabolites such as 1,4 benzoquinone (BQ), which can alter signaling pathways and affect chromosomal integrity. BQ has been shown to increase the activity of c-Myb, which is an important transcription factor involved in hematopoiesis, cell proliferation, and cell differentiation. The c-Myb protein has also been shown to increase topoisomerase IIα (Topo IIα) promoter activity specifically in cell lines with hematopoietic origin. Topo IIα is a critical nuclear enzyme that removes torsional strain by cleaving, untangling and religating double-stranded DNA. Since Topo IIα mediates DNA strand breaks, aberrant Topo IIα activity or increased protein levels may increase the formation of DNA strand breaks, leaving the cell susceptible to mutational events. We hypothesized that BQ can increase c-Myb activity, which in turn increases Topo IIα promoter activity resulting in increased DNA strand breaks. Using luciferase reporter assays in K-562 cells we demonstrated that BQ (25 and 37 μM) exposure caused an increase in c-Myb activity after 24 h. Contradictory to previous findings, overexpression of exogenous c-Myb or a polypeptide consisting of c-Myb's DNA binding domain (DBD), which competitively inhibits the binding of endogenous c-Myb to DNA, did not affect Topo IIα promoter activity. However, BQ (37 μM for 24 h) exposure caused a significant increase in Topo IIα promoter activity, which could be blocked by the overexpression of the DBD polypeptide, suggesting that BQ exposure increases Topo IIα promoter activity through the c-Myb signaling pathway.

1. Introduction

Due to its chemical and physical properties, benzene is an important industrial compound used as an organic solvent and as a precursor to manufacture various synthetic compounds such as rubber, plastics, dyes and pharmaceuticals [1]. However, exposure to benzene is linked to several toxicities and benzene is classified as an IARC Group I human carcinogen. Chronic exposure to benzene may lead to a variety of cancers such as leukemia, lung cancers and nasopharynegeal cancers, with leukemias being the most prevalent (reviewed in [1]).

While not fully understood, the toxicity of benzene is linked to the production of reactive metabolites formed during cytochrome P450 2E1 mediated bioactivation of benzene leading to a number of metabolites including phenol, benzene dihydrodiol and *trans*, *trans*-muconaldehyde [2,3]. These metabolites are further metabolized to produce hydroquinone and catechol, which can be metabolized in the bone marrow by myleoperoxidases to produce 1,4-benzoquinone (BQ) and 1,2-benzoquinone. Together these metabolites are thought to initiate benzene-induced toxicity through DNA alkylation, alteration of protein activity and/or reactive oxygen species (ROS) production.

We have been focusing on the hypothesis that benzene mediated leukemogenesis is mediated through alterations in an important hematopoietic signaling pathway involving the transcription factor c-Myb. The c-Myb gene encodes for a 78 kDa protein that regulates important biological processes such as proliferation, differentiation and apoptosis [4]. Given that studies have shown c-Myb up-regulation in various types of leukemia, lymophomas and cell lines derived from hematopoietic tumors (reviewed in [5]), disturbance in c-Myb signaling are thought to be involved in leukemogenesis.

Our previous *in vitro* studies have demonstrated that exposure to catechol, hydroquinone and BQ cause a time and concentration dependant increase in c-Myb activity and c-Myb phosphorylation without altering protein expression while exposure to phenol or benzene had no effect [6–8]. Furthermore, these effects can be abolished by treatment with the antioxidative enzyme superoxide dismutase [7]. Similar results were observed *in vivo* when

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pregnant mice were exposed to benzene during the critical period when embryonic hematopoiesis switches from the yolk sac to the liver (GD 10 and 11) [9]. These studies demonstrated that *in utero* exposure to benzene increased embryonic oxidative stress and embryonic c-Myb protein expression, both of which could be attenuated by antioxidant pre-treatment [9].

Given the proximity of a c-Myb binding site to the transcription start site of the enzyme topoisomerase II (Topo II) [10,11], one potential outcome of increased c-Myb activity may involve changes in Topo II protein expression. Topo II is an important homodermic ATP dependant endonuclease that modulates DNA topology by removing torsional strain during transcription, cell replication and cell division. Catalytically, Topo II covalently binds to double-stranded DNA and cleaves both DNA strands to form an "open cleaved complex". The binding of ATP stimulates the enzyme to pass another double-stranded duplex through the cleaved site to detangle the two duplexes. ATP hydrolysis then stimulates Topo II to religate the cleaved strands to regain its original confirmation. Although these transient strands breaks are tolerated by cells, any disturbance in Topo II protein activity or levels may lead to DNA damage. Topo II exists in two isoforms, Topo IIα and Topo IIβ. While both isoforms catalyze the same reaction, Topo IIα has been the most studied since it is usually the most abundant form in proliferating cells [12], and there is elevated expression of Topo IIα in tumor cells compared to control cells [13,14].

Previous studies have shown that c-Myb activates the Topo IIα promoter in HL-60 leukemia cells [15]. Interestingly, in this study, the role for c-Myb in Topo IIα expression appeared to be restricted to hematopoietic cells. Other studies have also demonstrated that a series of benzene metabolites block the function of Topo IIα *in vitro* [16,17] and *in vivo* in the bone marrow of benzene treated mice [18], supporting a role for this isoform in benzene-initiated toxicity. However, studies investigating the mechanism of Topo IIα inhibition (i.e. catalytic inhibition versus poison) by benzene metabolites failed to demonstrate that metabolites stabilized the Topo IIα/DNA cleavage complex [19]. Given that enhanced stabilization decreases religation of the nicked DNA strand resulting in increased strand breaks, these results suggested that inhibition of Topo IIα is not involved in benzene-initiated toxicity. However, these results have been disputed due to the presence of high concentrations of reducing agents in reaction buffers (which would interfere with the interaction of the metabolite with Topo IIα), and Lindsey et al. [20] showed that benzoquinone is a more potent Topo IIα poison than the anticancer drug etoposide, supporting a role for this enzyme in benzene-initiated leukemias. Thus, the bulk of evidence to date supports the hypothesis that benzene and its metabolites act as Topo IIα poisons, leading to increased Topo IIα/DNA cleavage complexes which prevent Topo IIα from completing the religation process. This would lead to increased DNA double strand breaks, which are known to be genotoxic.

These results suggest the possibility that increased activation of the c-Myb signaling pathway increases Topo IIα expression, potentially increasing Topo IIα-DNA cleavage complexes in the presence of benzene metabolites, leading to increased toxicity which may be an underlying molecular mechanism mediating leukemogenesis. The purpose of the current study was to investigate the effects of BQ on Topo IIα promoter activity in K-562 leukemic cells and determine whether any effects were dependent on c-Myb signaling.

2. Materials and methods

2.1. Cell culture and treatment

The human chronic myeloid leukemia K-562 cell line (CCL-243, ATCC, Manassas, VA, USA) was maintained at 37 °C/5% CO_2 in RPMI-1640 media containing L-glutamine which was supplemented with 10% fetal bovine serum, 23.4 mM sodium bicarbonate and 10 units/ml penicillin–streptomycin. All cell culture media reagents were obtained from Gibco™ Invitrogen Corporation (Burlington, ON, Canada) except for fetal bovine serum, which was acquired from Fisher Scientific (Whitby, ON, Canada). 1,4-Benzoquinone was purchased from Sigma and was at least 97% pure.

2.2. Transfections for luciferase assays

Plasmids expressing the *mim-1* promoter-linked to the luciferase gene (ΔEluc) and c-Myb were acquired from Dr. Scott Ness (Department of Molecular Genetics and Microbiology, University of New Mexico, NM, USA). The plasmid expressing the DNA binding domain of the c-Myb (DBD) was obtained from Dr. Giuseppe Raschella (Section of Toxicology and Biomedical Sciences, ENEA Research Center Casaccia, Italy). The plasmid expressing the Topo IIα promoter-linked to the luciferase gene (−620TOP2A-pGL3) was obtained from Dr. Susan P.C. Cole (Department of Pharmacology & Toxicology, Queen's University, ON, Canada). Plasmids expressing the renilla luciferase thymidine kinase (RLTK) and a control vector (pGEM) were purchased from Promega Inc. (Madison, WI, USA).

In transfection experiments, K-562 cells were plated at a density of 45×10^4 cells/ml per well in 24-well tissue culture plates (Day 0). Transfections were carried out 24 h later (Day 1) using FuGENE 6 Transfection Reagent (Roche Diagnostics Corporation, Indianapolis, IN, USA) according to the manufacturer's instructions. Twenty four hours after transfections (Day 2), cells were divided into two wells in order to unsure that cells did not become confluent, supplemented with fresh media and exposed to non-cytotoxic concentrations of BQ (0–37 μM) for an additional 24 h. Finally, after the exposure period (Day 3) the divided cells were recombined together and dual luciferase assays were performed.

To measure the effects of BQ on c-Myb activity, K-562 cells were transfected with 0.3 μg of ΔEluc plasmid DNA and 0.02 μg of RLTK plasmid DNA per well. To examine the effects of BQ on Topo IIα promoter activity, 0.2 μg of pGEM, 0.1 μg of −620TOP2A-pGL3 and 0.02 μg of RLTK plasmid DNA were transfected into each well. To examine the effects of overexpressing c-Myb or DBD on Topo IIα promoter activity, 0.1 μg of −620TOP2A-pGL3, 0.02 μg of RLTK and 0, 0.05, 0.1 or 0.2 μg of c-Myb or DBD expressing plasmids were transfected into cells. To examine the effects of BQ and DBD on Topo IIα promoter activity, 0.1 μg of −620TOP2A-pGL3, 0.02 μg of RLTK and 0, 0.05 or 0.2 μg of DBD expressing plasmids were transfected into each well. As described above 24 h after transfection, cells were split into two fractions, topped with more media and exposed to BQ (0–37 μM). In all cases, appropriate amounts of the empty vector pGEM were added to ensure equal amounts of DNA. Negative controls included cells that were transfected with empty vector alone or without any foreign DNA.

2.3. Dual luciferase assay

To determine luciferase activities, 48 h after transfection (Day 3), K-562 cells were recombined, washed twice in cold PBS and then lysed in 100 μl of 1× Passive Lysis Buffer (Promega Corp., Madison, WI). Dual luciferase activities were measured using 20 μl of the cell lysates using a nonproprietary dual luciferase assay [21] and a Lumat LB 9507 Variable Injector luminometer (Berthold Technologies GmbH & Co., Germany). The results are expressed as relative luciferase activity (RL1/RL2) where firefly luciferase readings were normalized to the renilla luciferase readings (internal control). To control for day-to-day differences between experiments, the relative luciferase activities of non-exposed controls were set to 100% and the relative luciferase activities for the experimental groups were expressed as a percentage of the values from non-exposed cells or from cells transfected with the empty vector pGEM.

2.4. Western immunoblotting

To investigate the effects of BQ on Topo IIα and c-Myb protein levels, K-562 cells (90×10^4) were plated into each well of a 6-well plate. After 48 h, cells were split in a 1:1 ratio, topped up with 2.332 ml of media and exposed to 0, 25 or 37 μM of BQ. After 24 h, the cells were pooled together, washed twice in cold PBS and lysed by suspension in 300 μl of RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 1 mM EDTA) containing protease inhibitors (1 mM PMSF, 1 mM benzamidine, 1 μM chymostatin, 1 μM leupeptin, 1 μM, antipain and 1 μM pepstatin A). The lysates were then sonicated on ice twice for 25 s using a Fisher Scientific Sonic Dismembrator 60 and then incubated on ice on a rocker for 30 min. The homogenates were then centrifuged at 15,000 × *g* for 10 min at room temperature and the supernatants were collected. Protein concentrations were measured using the Bio-Rad Laboratories Bradford Protein Assay (Mississauga, ON) and an Ultrospec 3100 Pro scanning spectrophotometer (Biochrom Ltd., UK). Samples containing equivalent amounts of protein were boiled in loading buffer (0.05 M Tris–HCl, 2.5 mM EDTA, 2% (w/v) sodium docecyl sulphate (SDS), 7% (v/v) glycerol, 0.002% (w/v) bromophenol blue) for 5 min.

The proteins were separated by SDS-PAGE on a 6% polyacrylamide gel and transferred to a polyvinylidene difluoride, carrier membrane (Milllipore Co, Bedford, MA). Subsequently, the membranes were blocked with 5% (w/v) skim milk dissolved in a Tris-based buffer containing Tween-20 (TBST – 25 mM Tris–HCl, 140 mM NaCl, 2 mM KCl and 0.05% (v/v) Tween-20). To probe for Topo IIα protein, membranes were incubated overnight at 4 °C with a 1:1000 dilution of 8D2 in 5% (w/v) milk-TBST