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Dibromoacetic acid induced Cl.Ly1 + 2/−9 T-cell apoptosis and activation of MAPKs signaling cascades

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ABSTRACT

Dibromoacetic acid (DBA), a haloacetic acid by-product of disinfection of drinking water, can cause many adverse effects in test animals, including immunotoxicity. However, the underlying molecular mechanism for the immunomodulatory effects remains unclear. The present study was undertaken to help in defining some potential mechanisms for this type of toxicity. Here, Cl.Ly1 + 2/−9 T-cells were exposed to varying levels of DBA and then several parameters, including cell survival, apoptosis, changes in mitochondrial potentials, and effects on select kinases (i.e., p38, ERK1/2, JNK1/2) were examined. The data showed that DBA significantly decreased Cl.Ly1 + 2/− 9 cell viability in a dose-related manner. DBA also induced apoptosis, a decrease in mitochondrial trans-membrane potential, and up-regulated the protein expression of cleaved caspase-3. Moreover, DBA increased the phosphorylation of all three mitogen-activated protein kinases (MAPKs) evaluated. Pre-treatment with specific p38, ERK1/2, and JNK1/2 inhibitors (SB203580, U0126, SP600125, respectively) attenuated the inducible phosphorylation events. DBA also induced up-regulation of mRNA levels of the MAPKs downstream transcription factors ATF-2 and Elk-1. When taken together, the results suggest that DBA could induce murine Cl.Ly1 + 2/− 9 T-cells apoptosis through mitochondria-dependent way, and activate the MAPKs pathways and downstream transcription factors ATF-2 and Elk-1.

1. Introduction

Haloacetic acids (HAAs) are the second-most prevalent class of disinfection by-products present in drinking water (Christman et al., 1983; Richardson, 2003). Five HAAs are regulated by the U.S. EPA, at a maximum contaminant level of 60 μg/l for the sum of bromoacetic acid (BA), dibromoacetic acid (DBA), chloroacetic acid (CA), dichloroacetic acid (DCA), and trichloroacetic acid (TCA) (U.S. Environmental Protection Agency, 2006). DCA and TCA are typically the dominant HAAs found in drinking water, but DBA can be higher in greater concentrations in waters when high bromide levels (> 50 μg/l) are present in the source waters. Moreover, higher levels of bromide in source waters generally result in a shift in speciation from DCA to DBA (Krasner et al., 2006; Plewa et al., 2010).

DBA was nominated to the National Toxicology Program by the United States Environmental Protection Agency for toxicity and carcinogenicity studies because of widespread human exposure and because its related chemical, DCA, was listed as possible carcinogens (Group C) (NTP, 2007; US EPA, 2011). So some rodent studies were carried out to test its potential for neurotoxicity, genotoxicity, reproductive and developmental toxicity, hepatotoxicity, and carcinogenicity. The results showed that DBA induced DNA damage in *Escherichia coli* PQ 37, cellular vacuolization in spinal cord gray matter, hepatocellular cytoplasmic vacuolization, delayed spermiation, retained spermatids; in two years study, DBA induced neoplasms in multiple sites including mononuclear cell leukemia, abdominal cavity mesotheliomas, hepatocellular adenoma or carcinoma, hepatoblastoma, alveolar adenoma or carcinoma(Giller et al., 1997; Bodensteiner et al., 2004; Moser et al., 2004; Tao et al., 2004; Melnick et al., 2007). DBA has also been shown to cause oxidative damage to DNA base in hepatocytes when it was given in drinking water to B6C3F1 mice (Parrish et al., 1996). In immunotoxicity studies, our previous studies showed that DBA caused thymic atrophy and splenomegaly in Balb/c mice exposed via daily intragastric administration at 5–50 mg DBA/kg for 28 days and inhibited T lymphocyte proliferation and induced T cells apoptosis; in vitro studies, we found that DBA induced thymus T cell apoptosis (at

Abbreviations: DBA, dibromoacetic acid; FITC, fluorescein isothiocyanate; PI, propidium iodide; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MMP, mitochondrial membrane potential; Rh123, Rhodamine 123; TBST, Tris-buffered saline containing 0.1% Triton X-100; PVDF, polyvinylidene difluoride; ATF-2, activating transcription factors 2

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doses of 20–40 μM) by blocking cell cycle progression, increasing intracellular calcium levels, and inducing Fas/FasL protein expression (Gao et al., 2008, 2016). And a significant decrease in the absolute number of CD^{4+} T-lymphocytes was observed at DBA dose of 125 mg/l in other study (Smith et al., 2010). These results indicated that T cell mediated cell immunity is a main target for DBA immunotoxicity testing. Based on above, ClLy + 2/9 cell line (cloned T cell line originated from C57BL/6TL + mouse spleens) was selected as our target in present study.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine-specific kinases, including extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinases 1and 2 (JNK1/2) family, and protein kinase of 38 kDa (p38) MAPK (Genestra, 2007; Johnson and Nakamura, 2007). Each MAPK can be activated by diverse extracellular stimuli including growth factors, chemicals, ultraviolet radiation, heat, synthesis inhibitors, metals, or foreign organisms (Davis, 2000; Brancho et al., 2003). Activation of MAPK triggers downstream signal cascades that can lead to inflammation, cell differentiation, or cell death (Kang et al., 2003; Hua and Reckhow, 2007). There are many targets of MAPK activities, including activating transcription factor-2 (ATF-2), Elk-1, c-Jun, etc. (Gupta et al., 1995; van Dam et al., 1995). Overall, various MAPK and their downstream transcription factors play a critical role in variety of cellular activities, including proliferation, differentiation or response to environmental stimuli (Carnello and Roux, 2011).

Mitochondria also can play a pivotal role in apoptotic pathways (Wang and Youle, 2016). Factors/processes that lead to disruption of mitochondrial membrane potential (Δψm) also give rise to a subsequent of cytochrome C organelle release. In general, depolarization of the membrane potential (dissipation of Δψm built across inner membrane) leads to a subsequent rupture of the outer membranes, and a non-selective release of IMS (intermembrane space) proteins (Liu et al., 1996). Among the latter is cytochrome C. Upon release, cytochrome C can - as an apoptotic protease activating factors (Apaf) - form a complex with apoptotic enzyme activators. The complex, in turn, activates caspase cascade reactions, including that with downstream caspase-3 and other caspases, ultimately leading to apoptosis (Liu et al., 2015; Zhu et al., 2015).

Because of the key roles of various MAPK in functions of many immune cells, and the interrelation between MAPK activities and both apoptosis and mitochondrial function, this study was undertaken to clarify potential mechanisms of effects of DBA previously seen in our laboratory. Here, studies were done to assess if DBA could induce apoptosis in Cl.Ly1 + 2/− 9 cells in the way it impacted isolated thymocytes. To that end, both mitochondrial dysfunction and caspase-3 activation in DBA-treated cells were examined in the context of any concurrent changes in levels of apoptosis induced. Moreover, if DBA could induce the activation of MAPK and related downstream factors, was also investigated in Cl.Ly1 + 2/− 9 cells.

2. Materials and methods

2.1. Reagents

A Cell Counting Kit-8 (CCK-8) was purchased from Promega (Madison, WI). Antibodies against MAPK proteins and caspase-3, as well as appropriate secondary antibodies, were purchased from Cell Signaling Technology (Danvers, MA). Inhibitors of MAPK (SP600125, SB203580 and U0126) were also purchased from Cell Signaling Technology, An Annexin V-FITC Apoptosis Detection Kit and Rh123 [cell-permeable cationic fluorescent dye readily sequestered by active mitochondria without toxic effects] were obtained from Becton Dickinson (San Jose, CA). RPMI 1640, fetal bovine serum (FBS), and Trizol were purchased from Invitrogen (Grand Island, NY). Glucose, pyruvate, IL-2, streptomycin, and penicillin were each purchased from Sigma (St. Louis, MO). DNA marker, a PrimeScript RT reagent Kit with DNA Eraser, SYBR and Premix Ex Taq II were each bought from TaKaRa (Takara Biotechnology, Dalian, China).

Table 1
The primer sequence of the target genes.

Target gene	Sequence(5′-3′)	Tm (°C)	size (bp)
Elk-1	Forward: GTGGTGAGTTCAAGTTGGTGGAT Reverse: GGGCAGTCTTCAGTGGAGCA	55.2	208
Atf-2	Forward: AAGATTGCCCTGTAACTGCC Reverse: TGCTGGATCGCTTCTGTATG	56.9	115
Jun	Forward: AGAACACGCTTCCCAGTGTC Reverse: GTTGCTGAGGTTGGCGTAG	59.8	156
β-Actin	Forward: GAGACCTTCAACACCCCTGC Reverse: ATGTCACGCACGATTTCCC	59.8	263

2.2. Cell culture

Cl.Ly1 + 2/− 9 cells (non-adherent cloned T-cell line derived from $C57BL/6TL^{+}$ spleen cells that produce IL-3, TCGF2, MCGF2 and BSF-1) were purchased from the National Platform of Experimental Cell Resources (Beijing, China). The cells, in complete RPMI 1640 (medium supplemented with 10% FBS, 5% glucose, 1 mM pyruvate, 100 U IL-2/ml, 100 μg streptomycin/ml and 100 U penicillin/ml) in a 5% CO_2-humidified 37 °C incubator (Thermo Forma, Marietta, OH), were cultured in 250-ml flasks. Cells were passaged at 80% confluency; cells were then diluted to 10^6 cells/ml in fresh medium and placed in new flasks.

2.3. Cell viability

Cells were cultured 18 h in 96-well plates (5×10^4 cells/well) and then treated with 0, 1, 5, 10, 20, or 40 μM DBA for 24, 48, or 72 h (four wells/dose/timepoint); control wells received only vehicle, the medium was taken as blank control. At that point, CCK-8 solution (10 μl) was added to each well, the plates were incubated 2 h at 37 °C for, and absorbance (450 nm) in each well was then measured in a micro-plate reader (Bio-Rad, Hercules, CA). Results obtained from three independent experiments, were expressed as percentage viable cells in comparison to control cells (average absorbance for these wells was normalized to 100%).

2.4. Flow cytometric analysis of apoptotic cells

For flow cytometric analysis, an Annexin V-FITC Apoptosis Detection Kit was used to determine phosphatidylserine externalization (early event in apoptosis) in DBA-treated cells. Specifically, cells were cultured at 37 °C for 18 h in 6-well plates (2×10^6/well) and then treated with 0.5–20 μM DBA for 24 h. Thereafter, the cells were collected from each well, washed with cold phosphate-buffered saline (PBS, pH 7.4), centrifuged, and resuspended in a final volume of 100 μl kit-provided binding buffer. Then cells then received 5 μl Annexin V and 5 μl PI (from kit) and were incubated at room temperature in the dark for 15 min. At the end of incubation, 400 μl binding buffer was added, and the cells were analyzed immediately in a FACSCalibur System (Becton Dickinson, San Jose, CA) using associated Cell Quest software. A minimum of 10,000 events/sample was acquired. At least three independent experiments were performed.

2.5. Mitochondrial membrane potential

Rh123, a cell-permeable cationic fluorescent dye readily sequestered by active mitochondria without toxic effects, was used to evaluate mitochondrial transmembrane potential (ΔΨm) (Perianayagam et al., 2005). In brief, cells were cultured 18 h at 37 °C in 6-well plates (2×10^6/well) and then treated with 1–20 μM DBA for 24 h. After