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Enhanced glutathione biosynthetic capacity promotes resistance to As^{3+}-induced apoptosis

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ABSTRACT

Trivalent arsenite (As^{3+}) is a known human carcinogen capable of inducing both cellular transformation and apoptotic cell death by mechanisms involving the production of reactive oxygen species. The tripeptide antioxidant glutathione (GSH) constitutes a vital cellular defense mechanism against oxidative stress. While intracellular levels of GSH are an important determinant of cellular susceptibility to undergo apoptotic cell death, it is not known whether cellular GSH biosynthetic capacity *per se* regulates As^{3+}-induced apoptosis. The rate-limiting enzyme in GSH biosynthesis is glutamate cysteine ligase (GCL), a heterodimeric holoenzyme composed of a catalytic (GCLC) and a modifier (GCLM) subunit. To determine whether increased GSH biosynthetic capacity enhanced cellular resistance to As^{3+}-induced apoptotic cell death, we utilized a mouse liver hepatoma (Hepa-1c1c7) cell line stably overexpressing both GCLC and GCLM. Overexpression of the GCL subunits increased GCL holoenzyme formation and activity and inhibited As^{3+}-induced apoptosis. This cytoprotective effect was associated with a decrease in As^{3+}-induced caspase activation, cleavage of caspase substrates and translocation of cytochrome c to the cytoplasm. In aggregate, these findings demonstrate that enhanced GSH biosynthetic capacity promotes resistance to As^{3+}-induced apoptosis by preventing mitochondrial dysfunction and cytochrome c release and highlight the role of the GSH antioxidant defense system in dictating hepatocyte sensitivity to As^{3+}-induced apoptotic cell death.

1. Introduction

Inorganic arsenic is a known human carcinogen associated with the development of cancers of the skin, bladder, lung, kidney and liver (Yoshida et al., 2004). Chronic exposure to low levels of trivalent arsenite (As^{3+}) also promotes cell proliferation and malignant transformation in various cultured cell models *in vitro* (Zhao et al., 1997; Huang et al., 1999; Achanzar et al., 2002; Chien et al., 2004; Sens et al., 2004). In contrast, acute exposure to high concentrations of As^{3+} induces apoptotic cell death (Bode and Dong, 2002). Paradoxically, although As^{3+} is a known carcinogen, As^{3+} in the form of arsenic trioxide (ATO) is a highly effective chemotherapeutic in the treatment of acute promyelocytic leukemia (Bode and Dong, 2002; Miller et al., 2002). While the molecular mechanisms mediating As^{3+}-induced transformation and apoptosis remain unclear, increased production of reactive oxygen species (ROS) has been implicated in both As^{3+}-induced genotoxicity and cytotoxicity (Kitchin and Ahmad, 2003). Mammalian cells possess a number of antioxidant defense mechanisms to protect against oxidative stress. The tripeptide antioxidant glutathione (GSH) is highly abundant and particularly effective means of protecting against oxidative injury (Griffith and Mulcahy, 1999). Interestingly, chronic exposure to sub-toxic doses of As^{3+} induces an adaptive response whereby cells become resistant to acute As^{3+}-induced apoptosis (Romach et al., 2000; Brambila et al., 2002; Chien et al., 2004; Somji et al., 2006). This acquired tolerance to As^{3+} toxicity occurs in concert with malignant transformation, suggesting that this may provide a selective growth advantage during As^{3+}-induced cellular transformation. The development of this resistant phenotype is associated with elevated intracellular GSH levels and increased expression of various detoxification and antioxidant enzymes, including enzymes involved in GSH biosynthesis, metabolism, and transport (Qu et al., 2001; Brambila et al., 2002; Chien et al., 2004; Coppin et al., 2008). While microarray studies have identified numerous gene products that could potentially mediate this apoptotic resistance (Chen et al., 2001a,b; Hamadeh et al., 2002), inhibition of GSH biosynthesis alone is sufficient to

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sensitize As^{3+}-transformed rat liver epithelial cells and human prostate cells to As^{3+}-induced apoptosis (Liu et al., 2001a; Brambila et al., 2002). These findings provide compelling evidence that up-regulation of GSH homeostasis contributes to acquired tolerance to As^{3+} during chronic As^{3+} exposure.

GSH homeostasis is dependent on GSH biosynthesis, utilization and export. The inability of most cells to import GSH highlights the importance of *de novo* GSH biosynthesis in maintaining GSH homeostasis. GSH biosynthetic capacity is dependent on several factors, including substrate availability and glutamate cysteine ligase (GCL) activity (Griffith and Mulcahy, 1999). GCL mediates the rate-limiting step in GSH biosynthesis and is a heterodimeric holoenzyme composed of a catalytic (GCLC) and a regulatory (GCLM) subunit (Griffith and Mulcahy, 1999). Cellular GCL activity is governed mainly by the relative levels of the GCL subunits which are highly regulated by transcriptional control mechanisms (Wild and Mulcahy, 2000; Franklin et al., 2009; Lu, 2009). We and others have demonstrated that both acute and chronic exposure to sub-toxic concentrations of As^{3+} coordinately induce GCL subunit expression resulting in increased cellular GCL activity and GSH (Li et al., 2002; Schuliga et al., 2002; Pi et al., 2003, 2008; Coppin et al., 2008; Thompson et al., 2009). There is also strong evidence that alterations in GSH levels play an important role in dictating cellular sensitivity to As^{3+}-induced apoptotic cell death (Bode and Dong, 2002; Miller et al., 2002). This is based on both comparative analyses and acute manipulation of cellular GSH levels. In this regard, increasing cellular GSH levels with the GSH precursor N-acetylcysteine (NAC) promotes cellular resistance to As^{3+}-induced apoptosis, while depletion of cellular GSH levels with the GCLC inhibitor buthionine sulfoximine (BSO) dramatically potentiates As^{3+}-induced apoptosis (Bode and Dong, 2002; Miller et al., 2002). Indeed, sufficiently elevating cellular GSH levels can abolish As^{3+}-induced apoptosis, while depletion of cellular GSH is capable of enhancing As^{3+} toxicity in rat liver epithelial cells by an order of magnitude (Shimizu et al., 1998; Bode and Dong, 2002; Miller et al., 2002). While these findings clearly demonstrate that cellular sensitivity to As^{3+}-induced apoptosis is inversely related to cellular GSH levels, they provide no information on the functional effects of altered GSH biosynthetic capacity *per se*.

GSH homeostasis is disrupted in most models of apoptotic cell death (Circu and Aw, 2008). This is mainly the result of the rapid depletion of cellular GSH levels due to extrusion of reduced GSH (Circu and Aw, 2008). However, altered GSH biosynthesis may also be a contributing factor as we have demonstrated that GCLC is a direct target for caspase-mediated cleavage during apoptosis (Siitonen et al., 1999; Pierce et al., 2000; Franklin et al., 2002, 2003). While it is still unclear how altered GSH homeostasis affects apoptotic cell death, GSH is thought to function at the level of the mitochondrion to prevent the loss of mitochondrial membrane potential and release of pro-apoptotic factors into the cytoplasm (Circu and Aw, 2008). Consistent with this working model, we have found that increased GCL activity resulting from GCL overexpression inhibits TNF-induced apoptosis by maintaining mitochondrial integrity and preventing the release of cytochrome c (Botta et al., 2004). While TNF induces cytochrome c release indirectly via Bid-mediated mitochondrial dysfunction, As^{3+} initiates cytochrome c release and activation of the apoptotic machinery by a direct effect on the mitochondrion (Larochette et al., 1999; Costantini et al., 2000). Based on the functional similarities of these cell death pathways, we believe that increased GSH biosynthetic capacity should also promote resistance to As^{3+}-induced apoptosis.

In this study, we directly examined whether increased expression of the GCL subunits and enhanced GSH biosynthetic capacity promotes cellular resistance to apoptotic cell death utilizing an established mouse liver hepatoma (Hepa-1c1c7) cell line overexpressing GCLC and GCLM (Botta et al., 2004). Hepa-1c1c7 cells are an excellent model to selectively examine the effects of increased GCL subunit expression and GCL activity as the relative levels of the GCL subunits, and not substrate availability, are limiting for GSH biosynthesis (Shertzer et al., 1995). Furthermore, Hepa-1c1c7 cells overexpressing the GCL subunits exhibit increased GSH biosynthetic capacity, but only a modest increase in cellular GSH content (Botta et al., 2004). GCL overexpression was found to suppress As^{3+}-induced translocation of cytochrome c to the cytoplasm and caspase activation, and inhibit As^{3+}-induced apoptosis. These findings provide proof-of-principle that up-regulation of GSH biosynthesis could mediate apoptotic resistance, promote cell survival and provide a selective growth advantage during chronic As^{3+} exposure.

2. Materials and methods

2.1. Reagents

$NaAsO_2$, 4′,6-diamidino-2-phenylindole (DAPI), and digitonin were purchased from Sigma Chemical Co. (St. Louis, MO). All reagents were prepared in either H_2O, sterile phosphate-buffered saline (PBS) or DMSO. z-VAD-fmk was from Bachem Bioscience (Torrance, CA) and Ac-DEVD-AMC, Ac-IETD-AMC, and Ac-LEHD-AMC were from Alexis Biochemicals (San Diego, CA).

2.2. Cell culture and treatments

Murine Hepa-1c1c7 cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM/F12 media (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were cultured at 37 °C in a humidified atmosphere of 95% air/5% CO_2. Hepa-1c1c7 cell lines expressing murine *GCLC* and *GCLM* expression vectors (Hepa-CR17) or a pMC1-neo vector (Hepa-V3) (Stratagene, La Jolla, CA) were established as previously described (Botta et al., 2004). Cells were seeded at $1.0–1.5 \times 10^6$ cells per 6-cm dish 18–24 h before treatment and all experiments were performed at 90–100% confluency.

2.3. Immunoblot analysis

Cells were harvested and lysed by a brief sonication on ice in TES/SB buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 250 mM sucrose, 20 mM boric acid, and 1 mM L-serine) containing 1× Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals, Indianapolis, IN). Lysates were clarified by centrifugation at 13,000 × *g* for 10 min at 4 °C and protein quantified by the Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of soluble protein (20 μg) were resolved on 10% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore, Bedford, MA). For analysis of GCL holoenzyme formation whole cell extracts were prepared in the absence of reducing equivalents and were resolved on native 10% PAGE gels in the absence of SDS. Samples for these studies were not boiled prior to gel loading and the gels were resolved in Tris/glycine buffer lacking SDS at 4 °C prior to transfer to PVDF membranes. Membranes were blocked in Tris-buffered saline/0.1% Tween-20 (TBST) containing 5% non-fat milk prior to incubation with primary antibody in TBST containing 0.5% milk. Membranes were probed for GCLC, GCLM (Thompson et al., 1999, 2009; Franklin et al., 2002, 2003), βActin (Sigma), pro-caspase-3, cytochrome c (BD Biosciences, San Diego, CA), Bid (R&D Systems, Minneapolis, MN), GADD153 and ATF-3 (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-mouse-HRP and anti-rabbit HRP secondary antibodies (Amersham Biosciences) were used at 1:5000 in TBST containing 0.5% milk. Antigen–antibody complexes were detected with Western Lightning Chemoluminescent Reagent (PerkinElmer, Boston, MA).

To detect translocation of cytochrome c from the mitochondria to cytosol, cells were fractionated by a rapid digitonin lysis procedure (Single et al., 1998). Cells were incubated on ice for 5 min in digitonin lysis buffer (DLB; 75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, and 250 mM sucrose) containing 20 μg digitonin per 1×10^6 cells. Lysates were centrifuged at 13,000 × *g* for 5 min at 4 °C. Supernatants were recovered and the pellets lysed by sonication in DLB and centrifuged as described above. Soluble protein (10 μg) from the fractions was resolved on 15% SDS-polyacrylamide gels and analyzed for cytochrome c by immunoblotting as described above.

2.4. GSH and GCL activity assays

Total GSH content (GSH + GSSG) was determined by a modification of the Tietze assay (Baker et al., 1990). Cell extracts were prepared by sonication in TES/SB as described above (Thompson et al., 1999; Franklin et al., 2002, 2003) and GSH levels were determined against a standard curve of GSSG and levels calculated per μg of soluble protein in the original cell extract. This value was utilized to determine the relative change in intracellular GSH levels compared to untreated samples. GCL activity was measured by a fluorescence-based NDA assay as described previously (White et al., 2003).