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Different ATM Signaling in Response to Chromium(VI) Metabolism via Ascorbate and Nonascorbate Reduction: Implications for *in* *Vitro* Models and Toxicogenomics

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Abstract

Background: Carcinogenic hexavalent chromium [Cr(VI)] requires cellular reduction for DNA damage. Cr(VI) metabolism by its principal reducer ascorbate (Asc) lacks Cr(V) intermediate, which is abundant in reactions with a minor reducer glutathione. Cultured cells are widely used in mechanistic studies of Cr(VI) toxicity; however, they typically contain <1% of normal Asc levels. Asc deficiency is also expected to diminish protection against reactive oxygen species.

Objectives: To assess how the presence of Asc in cells affects their stress signaling and survival responses to chromate.

Methods: We investigated the effects of Asc restoration in human lung H460 cells and normal human lung fibroblasts on the activation and functional role of ATM kinase, which controls DNA damage responses involving several hundreds of proteins.

Results: Cr(VI) treatments of standard cultures strongly activated ATM, as measured by its automodification at Ser1981 and phosphorylation of checkpoint kinase CHK2 and chromatin/transcription regulator KAP1 (TRIM28). Confirming the importance of activated ATM, its inhibition impaired replication recovery and clonogenic survival. In contrast, fully Asc-restored cells lacked ATM activation by Cr(VI) and ATM silencing produced no significant effects on p53 stabilization, apoptosis, replication recovery or clonogenic survival. Dose-dependent studies found a close correlation between ATM activation and the extent of Cr(VI) reduction by glutathione.

Conclusions: Asc restoration in cultured cells dramatically alters their stress responses to Cr(VI) by preventing activation of the oxidant-sensitive ATM network. We suggest that toxicogenomic

and other cell response-based approaches likely underestimate Cr(VI) genotoxicity when standard ATM-activating carcinogens are used as references.

Introduction

Hexavalent chromium [Cr(VI)] is a well-recognized human carcinogen that is found in the workplace of several millions of workers worldwide (ATSDR 2000). Environmental exposure to Cr(VI) has also raised questions about its potential adverse health effects in the general population (Salnikow and Zhitkovich 2008). Intracellular reduction of Cr(VI) to stable Cr(III) is responsible for the production of DNA-damaging products. Cr(VI) metabolism can generate variable amounts of Cr(V) and Cr(IV) intermediates, which is influenced by the nature of the reducer. Ascorbate (Asc) is the key reducer of Cr(VI) in cells *in vivo*, accounting for more than 90% of its metabolism (Suzuki and Fukuda 1990; Standeven and Wetterhahn 1991, 1992). The reduction of Cr(VI) by Asc generates Cr(IV) as the main intermediate at physiological conditions (Stearns and Wetterhahn 1994; Zhang and Lay 1996). Cr(VI) reactions with thiol-based secondary reducers yield reactive Cr(V) as the first intermediate (Borges et al. 1991; Bose et al. 1992). The final product of Cr(VI) reduction by all reducers is Cr(III), which forms several mutagenic Cr-DNA adducts (Zhitkovich 2005). Human and nonhepatic rodent cells in standard cultures contain either undetectable or low micromolar concentrations of Asc (Reynolds et al. 2007, 2012) in contrast to its 1-3 millimolar amounts in the main tissues *in vivo* (Bergsten et al. 1990; Kojo 2004). Consequently, metabolism of Cr(VI) in cultured cells is dominated by the most abundant thiol glutathione (GSH) (Zhitkovich 2005), which yields Cr(V) species that can cause oxidative damage via direct or Fenton-like reactions (Sugden and Stearns 2000; Sugden et al. 2001). Findings in cultured cells commonly guide the design and interpretation of expensive animal studies and are used in the determination of the mode of action for the regulatory purposes. Thus, it is critical to ensure that *in vitro* models adequately recapitulate the main metabolic processes for Cr(VI) in tissues.

The main signaling network activated by oxidants and other DNA breakage-inducing agents is initiated by ATM kinase that regulates phosphorylation of more than 1000 proteins, including stress-sensitive transcriptional factors, and consequently, orchestrates changes in all major cellular processes such as DNA repair, chromatin remodeling, gene expression, cell cycle, cell death, metabolism and others (Ditch and Paull 2012; Shiloh and Ziv 2013). ATM can also be activated by chromatin injury (Kaidi and Jackson 2013) and direct oxidation of its cysteines (Guo et al. 2010). Given this broad importance of ATM in regulation of cellular responses to DNA damage and oxidants, examination of activation of this kinase by Cr(VI) has a potential to uncover critical signaling and survival mechanisms. Asc-deficient human cells treated with Cr(VI) showed some evidence of activated ATM (Ha et al. 2003; Hill et al. 2008; Wakeman et al. 2004), which has not yet been assessed functionally in the isogenic systems or verified in cells with physiological amounts of Asc.

In this work, we investigated activation of ATM signaling and its significance in Cr(VI)-treated human cells with and without restoration of physiological concentrations of Asc. We found that Cr(VI) caused a robust stimulation of ATM in Asc-deficient cells, which increased their replication recovery and long term-survival. In contrast, all of the tested ATM-dependent responses were absent in Asc-restored cells. Thus, the presence of Asc dramatically altered Cr(VI)-induced cell stress responses, which excluded a canonical DNA damage signaling by ATM. Lack of ATM activation under physiological conditions of Cr(VI) metabolism has important implications for the use of *in vitro* models in Cr(VI) research and the assessment of Cr(VI) genotoxicity by toxicogenomic approaches *in vivo*.

Methods

Cells and treatments. All cell lines were purchased from the American Type Culture Collection. H460 cells were grown in RPMI-1640 medium with 10% serum under 95% air/5% CO₂. Normal IMR90 fibroblasts proliferate better under physiological oxygen tension and they were cultured in the atmosphere of 5% O₂/5% CO₂ using DMEM medium and 10% serum. Cells were treated in the complete growth media for 3 hr with K₂CrO₄ [Cr(VI)] and for 1 hr with camptothecin (CPT). ATM inhibitors KU60019 (ATM-i1, 1 μM) and KU55933 (ATM-i2, 5 μM) were added simultaneously with Cr(VI). In a time-course study of CHK2 phosphorylation, H460 cells were treated with Cr(VI) for 3 hr and collected for protein extraction at 0, 2, 4 and 8 hr post-exposure. Cells were depleted of GSH by preincubation with 0.1 mM buthionine-sulfoximine (BSO) for 24 hr before the addition of Cr(VI) (DeLoughery et al. 2014).

Asc restoration in cells. Cells were incubated for 90 min with dehydroascorbic acid and cellular Asc was quantified using 1,2-diamino-4,5-dimethoxybenzene (Reynolds and Zhitkovich 2007). Cellular volumes were calculated from forward scatter profiles generated by flow cytometry (FACSCalibur, BD Biosciences).

shRNA. pSUPER-retro based vectors were used to produce stable knockdowns of ATM and CHK2. Targeting sequences for ATM and CHK2 were 5'-GATACCAGATCCTTGGAGA-3' and 5'-AATGTGTGAATGACAACACTACT-3', respectively. Oligonucleotides and the linearized vector were incubated with T4 ligase overnight followed by the transformation of the plasmid products into *E. coli* cells. The vectors were packaged into the viral particles by cotransfection with MoMuLV gag-pol and VSVG-encoding plasmids into 293T cells. Virus-containing media

was collected 48 hrs after transfection, filtered and added to H460 cells overnight. Infected cells were selected and continuously maintained in the presence of 1.5 $\mu\text{g}/\text{mL}$ puromycin.

Western blotting. Protein extracts were prepared by boiling of cells for 10 min in 2% SDS buffer (2% SDS, 50 mM Tris-HCl pH 6.8, 10% glycerol). For proteins smaller than 100 kDa, cell extracts were separated on 10% SDS-PAGE gels and then electroblotted onto PVDF membranes by a semi-dry transfer (PierceG2 Fast Blotter). For detection of ATM and phospho-ATM, proteins were separated on 6% gels and electroblotted onto PVDF membranes overnight by a cold wet transfer. Primary antibodies were from Cell Signaling Technology (phospho Thr68-CHK2, PARP, cleaved caspase 7, CHK2, phospho S15-p53), Bethyl (phospho Ser824-KAP1), BD Biosciences (MSH6), Santa Cruz Biotechnology (ATM, p53), Abcam (phospho Ser1981-ATM) and Sigma-Aldrich (γ -tubulin).

Scoring of replicating cells. IMR90 cells were seeded on human fibronectin-coated coverslips and Asc-preloaded on the next day. After Cr(VI) exposure for 3 hr, cells were allowed to recover for 24 hr before labeling with 10 μM EdU for 1 hr. ATM-i1 (1 μM KU60019) was present during and after exposure to Cr(VI). Cells were fixed with ice-cold methanol for 10 min and EdU incorporation was visualized by the DNA Click-iT AlexaFluor 488 Imaging Kit (Molecular Probes). Images of DAPI/EdU-stained slides were analyzed using SpotAdvanced 5.1.23 software.

Cell viability. Cr(VI) cytotoxicity was monitored by the measurements of metabolic activity using the CellTiter-Glo luminescent kit (Promega). Cells were seeded into 96-well optical bottom plates and allowed to attach overnight before Cr(VI) treatments. Cytotoxicity was assessed at 48 hr post-Cr(VI) exposure. Each viability experiment included 5-6 wells per dose.

Clonogenic survival. Cells were seeded onto 60-mm dishes and treated with Cr(VI) for 3 hr on the next day. After growth for 7-8 days, colonies were fixed with methanol and stained with a Giemsa solution. Each clonogenic experiment included 3-4 dishes per dose.

Uptake of Cr(VI). Cellular chromium was measured by graphite furnace atomic absorption spectroscopy (GF-AAS) (Messer et al. 2006). After removal of Cr(VI)-containing media, cells were washed twice with warm PBS and then detached with a trypsin-EDTA solution (Gibco 15400-054). Cells were collected at 800xg for 5 min at 4°C and washed two times with cold PBS. Cellular Cr was extracted by hot nitric acid and quantified by GF-AAS (AAAnalyst600 Atomic Absorption Spectrometer).

Cr(VI) reduction. The rate of Cr(VI) reduction was measured at 37°C by recording absorbance at 372 nm every 20-30 s. Reactions contained 50 mM MOPS (pH 7.0), 100 mM NaCl, 50 µM Cr(VI) and various reducers such as thiols (3 mM GSH+0.2 mM Cys), Asc (0.2-0.6 mM) and mixtures of thiols with Asc.

Statistics. Differences between the groups were evaluated using two-tailed, unpaired *t*-test. Error bars in Figures are SD values.

Results

ATM activation in standard culture of H460 cells. We chose H460 human lung epithelial cells as our main cellular model for the determination of Cr(VI) effects on ATM pathway. These cells display normal DNA damage signaling to a classic ATM activator ionizing radiation (Zhang et al. 2006) and contain wild-type stress-sensitive transcriptional factor p53, which showed robust upregulation in response to genotoxic and nongenotoxic carcinogens (Wong et al. 2012; Wong et

al. 2013). Lung is a main target of carcinogenic effects in Cr(VI)-exposed workers (Salnikow and Zhitkovich 2008), making H460 cells a histologically relevant model. Our standard H460 cultures contained 7 ± 4 μ M cellular Asc (n=6), which is less than 1% of its normal concentration in the human lung (Slade et al. 1985). To monitor ATM activation, we examined phosphorylation status of three well-characterized ATM targets: ATM autophosphorylation at Ser1981, checkpoint kinase CHK2 at Thr68 and transcription/chromatin regulator KAP1 at Ser824 (Shiloh and Ziv 2013). We found that all three proteins showed elevated phosphorylation after Cr(VI) exposure (Figure 1A). Addition of two selective ATM inhibitors (KU60019, KU55933) blocked Cr(VI)-induced phosphorylation, confirming its ATM-dependence. A less specific ATM target, Ser15 in the transcriptional factor p53, also showed a strongly increased phosphorylation by Cr(VI), which was partially dependent on ATM as evidenced by the suppressive effects of its kinase inhibitors, particularly for the lower Cr(VI) concentration (Figure 1A). A time-course analysis of CHK2 phosphorylation found that ATM activity was the highest in cells collected immediately after Cr(VI) exposure, which was followed by a gradual decline over several hours of recovery (Figure 1B). Induction of apoptosis, assayed by PARP cleavage, did not occur until 8 hr post-Cr, indicating that CHK2 phosphorylation and by extension, ATM activation, were not triggered by apoptotic DNA fragmentation. ATM was important for protection against Cr(VI) toxicity, as evidenced by a significantly decreased clonogenic survival of cells in the presence of its kinase inhibitor (Figure 1C).

Effects of Asc restoration on ATM activation. To examine the effects of Asc, we elevated its levels in H460 cells to 2.8 ± 0.3 mM (n=3). Asc-restored (Asc+) cells had on average 1.37-times lower accumulation of Cr(VI) ($p < 0.001$) for its three tested concentrations (Figure 2A). The

differences in uptake were also statistically significant for individually analyzed 5 μM ($p < 0.01$) and 10 μM ($p = 0.02$) Cr(VI) concentrations. The observed decrease in the entry of Cr(VI) could be caused by its extracellular reduction to impermeable Cr(III) by leaked cellular Asc. Mock-preloaded cells showed expected increases in phosphorylation levels of all three ATM targets by Cr(VI) but, in striking contrast, no responses were detected in Asc⁺ cells (Figure 2B). This inhibitory effect of cellular Asc on ATM signaling was clearly much more dramatic than its modest decrease of Cr(VI) uptake. Asc⁺ cells also displayed a substantially lower stress signaling targeting p53, as monitored by its protein and Ser15 phosphorylation levels (Figure 2C). The remaining increases of p53 readouts in Asc⁺ cells likely reflect the contribution of ATM-independent signaling, as evident from the inability of ATM inhibitors to completely suppress p53-Ser15 phosphorylation in Asc-deficient cells (Figure 1A). Consistent with the lack of significant ATM activation, the addition of its inhibitors had no effect on cytotoxicity and clonogenic lethality of Cr(VI) in Asc⁺ cells (Figure 2D,E). Unlike Cr(VI), phosphorylation of ATM targets CHK2-Thr68 and KAP1-S821 by the topoisomerase I poison camptothecin was not altered by Asc restoration (Figure 2F), indicating that Asc does not act as a general suppressor of ATM signaling.

Next, we employed genetic approaches to further explore a potential involvement of ATM signaling in cytotoxic responses to Cr(VI) in Asc⁺ cells. We constructed stable knockdowns of ATM and its main transducer kinase CHK2 and tested their effects on the activation of the transcriptional factor p53 and apoptotic responses. Similar to parental H460 cells with restored Asc, the shRNA-expressing lines showed no increases in phosphorylation of the ATM targets CHK2 and KAP1 at the end of Cr(VI) exposures (Figure 3A). Consistent with the inactivity of

the ATM pathway, upregulation of p53 protein and its Ser15 phosphorylation by Cr(VI) were unaffected by ATM or CHK2 depletion (Figure 3B). In cells collected at 24 hr post-exposure, Cr(VI)-treated samples showed activation of apoptosis as measured by PARP cleavage and the formation of cleaved (active) caspase-7. ATM knockdown produced no effects on the apoptotic responses or activation of p53 in these samples (Figure 3C,D). CHK2-depleted cells had slightly higher amounts of cleaved caspase-7 for 4 μ M but not 8 μ M Cr(VI), although this kinase usually plays a proapoptotic role in ATM signaling (Stracker et al. 2009). It is possible that a loss of CHK2 altered activation of a related kinase CHK1, which can respond to Cr-DNA adducts. Taken together, these results indicate that ATM does not play a significant role in the apoptotic effects of Cr(VI) in H460 cells containing physiological levels of Asc.

Dose-dependent effects of Asc. To better understand how Asc suppresses ATM activation, we examined dose-dependent effects of this reducer in cells and analyzed its rates of Cr(VI) reduction. Small thiols GSH and Cys are responsible for Asc-independent reduction of Cr(VI) in cells (Zhitkovich 2005). We have previously determined that H460 cells contained approximately 3 mM GSH (Reynolds et al., 2012) and 0.2 mM Cys (DeLoughery et al., 2014). Figure 4A shows that the rate of Cr(VI) reduction by 0.3 mM Asc was 2.4-times faster than that by a ten-fold higher concentration of cellular thiols (3 mM GSH + 0.2 mM Cys). Despite a clear kinetic superiority of Asc, its mixture with thiols yielded an approximately additive increase in Cr(VI) reduction, indicating a continuing independent contribution of GSH/Cys. Based on a series of additional kinetic studies, we calculated the contribution of the physiological mixture of thiols (3 mM GSH + 0.2 mM Cys) in the presence of different concentrations of Asc. To see whether the extent of Cr(VI) metabolism by thiols correlates with the degree of ATM activation,

we next examined induction of CHK2 phosphorylation by Cr(VI) in H460 cells preloaded with different Asc concentrations. Since Asc leaks from cells in culture (Reynolds and Zhitkovich 2007), we first measured the amount of Asc in H460 cells at the end of our standard 3 hr-long incubations. We found that cells lost approximately 50-60% of Asc without Cr(VI) and 70-80% in the presence of 8 μ M Cr(VI) (Figure 4B). There was a statistically borderline trend for a larger depletion of Asc by Cr(VI) in the cells preloaded with the lowest dose ($p=0.067$ for 0.32 mM samples+Cr *versus* 1 mM samples+Cr). The loss of Asc in culture is not limited to established cell lines, as freshly purified human lymphocytes also lost their Asc during *in vitro* incubation (Bergsten et al. 1990). It is currently unclear whether cells lose Asc through passive or active mechanisms and what factors regulate this process. It is possible that the absence of Asc in the extracellular medium triggers active efflux of cellular Asc to establish antioxidant defense of the outer layer of the plasma membrane. Our attempts to maintain cellular Asc levels using the continuous presence of reduced and/or oxidized Asc so far have been unsuccessful, possibly due to oxidation of Asc in the iron-rich culture media. Using the initial and final concentrations of cellular Asc, we calculated its mid-exposure levels and included them on the X-axis in dose-dependent analyses of phospho-CHK2 induction by Cr(VI) (Figure 4C). The amounts of phospho-CHK2 and the percentage of Cr(VI) reduction by thiols showed similar inverse dose-dependence on Asc (Figure 4D), suggesting that a shift to the thiols-independent Cr(VI) metabolism was a likely cause of ATM-suppressive effects of cellular Asc. A unique feature of Cr(VI) reduction by GSH and Cys is a direct production of Cr(V) intermediate via the initial one-electron transfer reaction (Zhitkovich 2005). Depletion of GSH increases abundance of Cr(V) due to its extended stability under the lower concentrations of cellular reducers (DeLoughery et

al. 2014). The amount of phospho-CHK2 by Cr(VI) was also increased in GSH-depleted cells (Figure 4E), which is consistent with the ability of Cr(V) to promote ATM activation.

ATM responses in normal human lung cells. To test the effects of cellular Asc in normal human cells, we employed IMR90 lung fibroblasts. These cells showed large losses of Asc after 3-hr long incubations, retaining only approximately 15% of the initially loaded amounts (Figure 5A). In subsequent experiments, we preloaded IMR90 with the initial concentration of 3.4 mM Asc, which at the midpoint of our standard 3-hr long exposures drops to 1.3 mM. Uptake of Cr(VI) by IMR90 cells was unaffected by their preloading with Asc (Figure 5B). Similar to H460 cells, Cr(VI) induced a robust phosphorylation of ATM targets in control IMR90 ($12 \pm 6 \mu\text{M}$ cellular Asc) but these responses were prevented by Asc restoration (Figure 5C,D). The ability of Cr(VI)-treated Asc⁺ IMR90 cells to continue replication, as scored by the percentage of S-phase cells at 24 hr post-exposure, was not altered by the inhibition of ATM by KU60019 (Figure 5E), further supporting the lack of a biologically significant activation of this kinase. In agreement with the western blotting results on the activation of ATM in Asc-deficient IMR90, the addition of the same ATM inhibitor to these cells increased replication-inhibitory effects of Cr(VI) (Figure 5F). To test whether Asc restoration changes cellular physiology, we examined stress signaling in IMR90 cells treated with Cr(VI) at 6 hr post-Asc loading. At this time, cells retained only $3.2 \pm 0.2\%$ of the initially loaded Asc (3.4 mM Asc in freshly loaded cells). We found that a loss of cellular Asc resulted in the re-establishment of ATM activation and p53 phosphorylation by Cr(VI) without any changes in metal accumulation (Figure 5G,H). Thus, suppression of ATM signaling by Asc requires its presence during Cr(VI) exposure, which again points to Cr(VI) metabolism as the primary target of Asc-induced effects.

Discussion

Reducer effects in ATM activation by Cr(VI). Our results showed that Cr(VI) was capable of a robust activation of the apical DNA damage-responsive kinase ATM in normal and transformed human lung cells grown under the standard Asc-deficient conditions. Inhibition of ATM activity in these cells decreased their clonogenic survival and replication recovery following Cr(VI) exposure, demonstrating a toxicological importance of ATM signaling. In a striking contrast, we found no biochemical or functional evidence for ATM activation by Cr(VI) in Asc-restored cells. ATM activity toward its major target CHK2 was already decreased by 50% at approximately 1:20 ratio of cellular Asc to GSH and it was completely suppressed at 1:4 ratio. *In vivo* cellular Asc and GSH are present from 0.5:1 to 2:1 ratio (Kojo 2004; Meister 1994). In the absence of Asc, Cr(VI) is reduced in cells by thiols (primarily by GSH) via one-electron transfer reactions, which results in the production of reactive Cr(V) (Zhitkovich 2005). Asc initiates Cr(VI) reduction via two-electron transfer, yielding Cr(IV) as the sole intermediate (Stearns and Wetterhahn 1994; Zhang and Lay 1996). Studies with redox-sensitive probes *in vitro* and in cells showed that Cr(V) but not Cr(IV) species can act as oxidants (DeLoughery et al. 2014). Taken together with the observed close correlation of thiol-mediated reduction and CHK2 phosphorylation (Figure 4D), this suggests that Cr(V) intermediates were likely responsible for ATM activation by Cr(VI) in Asc-deficient cells. The absence of ATM activation in Asc⁺ cells is consistent with a suppression of oxidative DNA damage by Cr(VI) in Asc-replete cells (Reynolds et al. 2012). However, cellular Asc does not diminish all forms of Cr(VI) genotoxicity, as uptake-normalized formation of Cr-DNA adducts, DNA-protein crosslinks or DNA and chromosomal breaks have been found to be either similar or even higher in Asc-restored cells (Macfie et al. 2010; Reynolds and Zhitkovich 2007; Reynolds et al. 2007, 2009).

Oxidants can trigger ATM activity either as a result of the production of DNA double-strand breaks (Shiloh and Ziv 2013) or ATM dimerization via oxidation of its cysteines (Guo et al. 2010). A characteristic feature of ATM activated by Cys oxidation is the lack of phosphorylation of chromatin-bound targets, such as KAP1. We observed phosphorylation of both KAP1 and a soluble protein CHK2, suggesting that DNA damage was involved in stimulating ATM activity. Genetic approaches revealed a significant formation of oxidative DNA damage by Cr(VI) in Asc-deficient but not in Asc-restored cells (Reynolds et al., 2012). Thus, it is likely that oxidative DNA damage played a major role in ATM activation during non-ascorbate reduction of Cr(VI). Cr(VI) metabolism also causes oxidation of protein thiols (Myers, 2012) and some direct oxidation of ATM cysteines triggering kinase activity cannot be ruled out. In addition to the formation of reactive Cr(V) during one-electron reduction of Cr(VI), oxidative stress in Asc-deficient cells is further promoted by the generation of GS• radicals, elimination of which is associated with the formation of superoxide and H₂O₂. Asc is a preferred cellular scavenger for various reactive oxygen species and its radical does not form toxic byproducts (Winterbourn 2008).

Toxicological implications. ATM is a major coordinator of DNA damage responses, which directly or via its downstream kinases and other transducers controls phosphorylation status of more than 1000 proteins in cells exposed to oxidants (Ditch and Paull 2012; Shiloh and Ziv 2013). ATM-dependent signaling network regulates approximately 350 proteins involved in gene expression control, including several major transcriptional factors such as p53, ATF1, E2F1 and NF-κB. A broad spectrum of protein targets and cellular functions controlled by ATM makes this kinase a dominant regulator of stress responses to many DNA damaging agents. The

inability of Cr(VI) to activate ATM in Asc-restored cells indicates that despite its clear evidence of genotoxicity, such as activation of nucleotide excision repair, Cr-DNA adduction and DNA and chromosomal breakage even at low exposure levels (DeLoughery et al. 2015, Reynolds et al. 2007, 2009; Zhitkovich 2011), Cr(VI)-induced signaling is expected to lack many typical features of DNA damage responses. Supporting this suggestion are the findings that Cr(VI)-induced apoptosis in Asc-restored cells was p53-independent (Reynolds and Zhitkovich 2007) and histone H2AX phosphorylation by Cr(VI)-induced DNA breaks was mediated by ATR, not ATM kinase (DeLoughery et al. 2015). DNA double-strand breaks by Cr(VI) are selectively formed in euchromatin of Asc-restored cells (DeLoughery et al. 2015). Although ATM is typically a central regulator of cellular responses to DNA double-strand breaks, it is dispensable for repair of euchromatic breaks (Goodarzi et al. 2008; Rass et al. 2013), explaining the observed lack of functional importance of ATM for recovery after Cr(VI) exposures. Stress signaling by DNA-protein crosslinks, which is another form of Cr-DNA damage, is also dependent on ATR kinase, not ATM (Wong et al. 2012).

Classification of Cr(VI) as a genotoxic or nongenotoxic carcinogen has a major impact on its risk assessment, calling for the use of linear or nonlinear low-dose extrapolation models, respectively (Zhitkovich 2011). One of the current approaches for understanding of mechanisms of toxicity or the mode of carcinogenic action relies on the characterization of perturbed cellular pathways, which is most commonly done by examination of gene expression profiles. Transcriptome analyses of small intestine of mice exposed to Cr(VI) in drinking water have not detected a classic genotoxic signature, which was taken as evidence of nongenotoxic mode of action in this tissue (Kopec et al. 2012; Thompson et al. 2012). Alternatively, the inability of these studies to

detect typical DNA damage-associated transcriptome changes can be explained by our findings on the absence of ATM activation during Cr(VI) metabolism by its principal physiological reducer Asc.

Conclusions

A near absence of Asc in cells maintained under the standard culture conditions results in the distorted stress responses to Cr(VI) due to its metabolism by secondary reducers. Cr-DNA damage in cells with restored Asc does not engage a classic genotoxic signaling relying on ATM kinase. We suggest that the assessment of Cr(VI) genotoxicity *in vivo* by transcriptome profiling and other indirect analyses should include reference carcinogens with ATM-independent DNA damage responses in the tested tissues.

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Figure Legends

Figure 1. Activation of ATM signaling in H460 cells without Asc restoration. Cells were treated with Cr(VI) for 3 hr in the absence and presence of ATM inhibitors (ATM-i1 - 1 μ M KU60019, ATM-i2 - 5 μ M KU55933). **(A)** ATM-dependent protein phosphorylation immediately after Cr(VI) exposure. **(B)** Western blots of cells at different times after Cr(VI) exposure. **(C)** Clonogenic toxicity of Cr(VI) in the presence and absence of 1 μ M ATM-i1 (**- $p < 0.01$ relative to cells without inhibitor, $n=3$).

Figure 2. ATM responses in Asc-restored H460 cells. Control and Asc-preloaded cells (+Asc) were treated with Cr(VI) for 3 hr and then immediately collected. Inhibitors: ATM-i1 - 1 μ M KU60019, ATM-i2 - 5 μ M KU55933. **(A)** Cr(VI) uptake ($n=3$). **(B)** Suppression of ATM activation by Asc restoration. **(C)** Impact of cellular Asc on p53 upregulation by Cr(VI). **(D)** Effect of ATM inhibitors on cell viability (72 hr post-Cr measurements, $n=4$) and **(E)** clonogenic survival ($n=2$). Data in panels D and E are means \pm SD. **(F)** Phosphorylation of ATM targets in cells treated with camptothecin (CPT).

Figure 3. Impact of ATM and CHK2 knockdowns on p53 and apoptotic responses in Asc-restored H460. **(A)** ATM-related phosphorylation in cells collected immediately after 3 hr Cr(VI) exposures. **(B)** Activation of p53 in cells immediately after Cr(VI) exposure. **(C,D)** Western blots of cells collected at 24 hr post-Cr.

Figure 4. Dose-dependent effects of Asc on CHK2 phosphorylation and Cr(VI) reduction. **(A)** Cr(VI) reduction *in vitro* by 0.3 mM Asc, 3 mM GSH + 0.2 mM Cys, and a mixture of all three reducers. **(B)** Loss of Asc from H460 cells during 3 hr incubation with and without 8 μ M Cr(VI). Cells were preloaded with 0.32, 0.64 and 1 mM Asc (0 hr) and analyzed for Asc after 3 hr incubations without (3 hr) and with Cr(VI) (3hr- Cr). **(C)** CHK2 phosphorylation in H460 cells preloaded with different concentrations of Asc prior to Cr(VI) exposure for 3 hr. Calculated mid-exposure Asc concentrations are shown. **(D)** Comparison between the amounts of Cr(VI)-induced phospho-CHK2 and the contribution of thiols to Cr(VI) reduction at different Asc levels. Cells were preloaded with Asc and treated with Cr(VI) as in panel C. Data are means \pm SD for

three independent experiments. **(E)** Effect of GSH depletion on CHK2 phosphorylation. H460 cells were pretreated with 0.1 mM BSO for 24 and then exposed to 8 μ M Cr(VI) for 3 hr.

Figure 5. ATM activation in IMR90 normal human lung cells. Control and Asc-restored (+Asc, 3.4 mM Asc) cells were treated with Cr(VI) for 3 hr. Bar and line graphs show means \pm SD, n=3. **(A)** Asc levels in cells before and after 3 hr incubations in the regular medium. **(B)** Uptake of Cr(VI) by control and Asc-restored (+Asc, 3.4 mM Asc) cells. **(C)** Western blots of cells collected immediately after Cr(VI) exposure. Images of control and +Asc samples are from the same blots, which were cropped to remove unrelated intervening lanes. **(D)** Western blots of +Asc cells collected at 3 hr post-exposure to Cr(VI). **(E)** Percentage of +Asc cells in S-phase at 24 hr post-exposure. ATM-i (1 μ M KU60019) was present during Cr(VI) exposure and subsequent 24 hr incubations. **(F)** As panel E, except that cells were treated with Cr(VI) without Asc restoration. **(G)** ATM activation and p53 phosphorylation in cells treated with Cr(VI) at 6 hr after Asc loading (3 hr Cr exposure, immediate collection). **(H)** Cr(VI) uptake by cells treated at 6 hr post-Asc loading. In panels G and H, +Asc cells contained 0.110 \pm 0.006 mM Asc at the start of Cr exposures.

Figure 1.

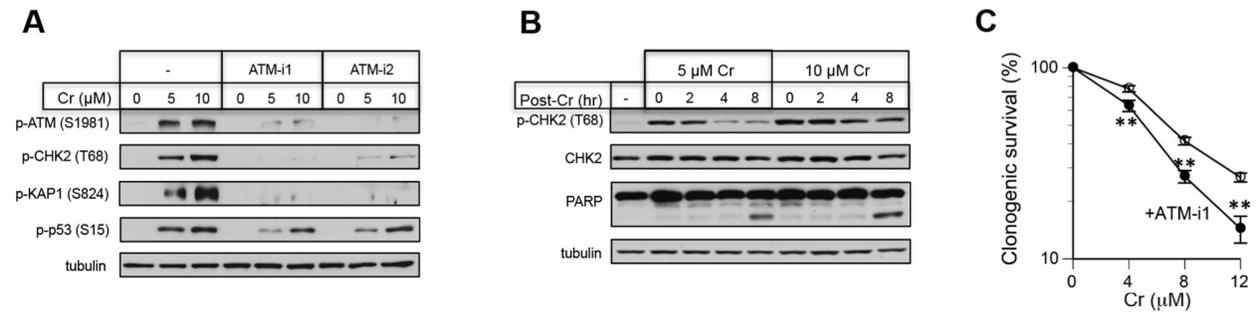


Figure 2.

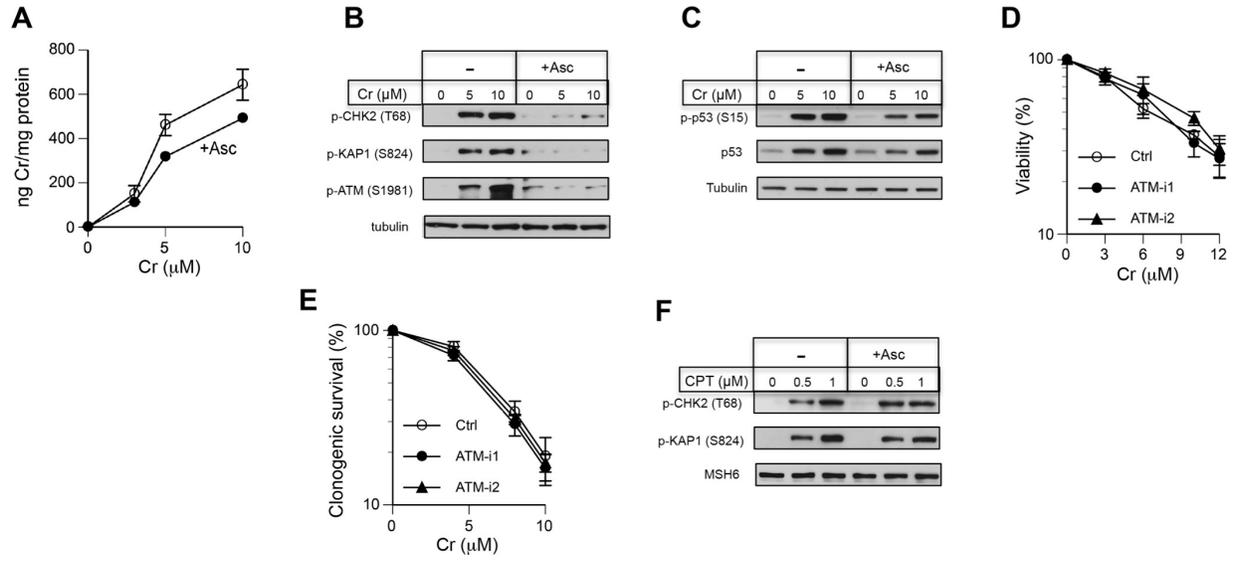


Figure 3.

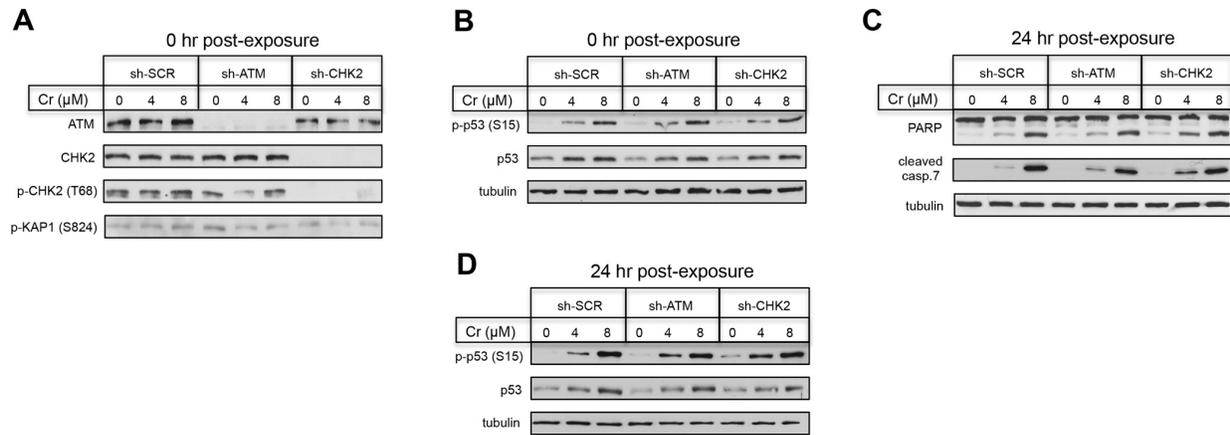


Figure 4.

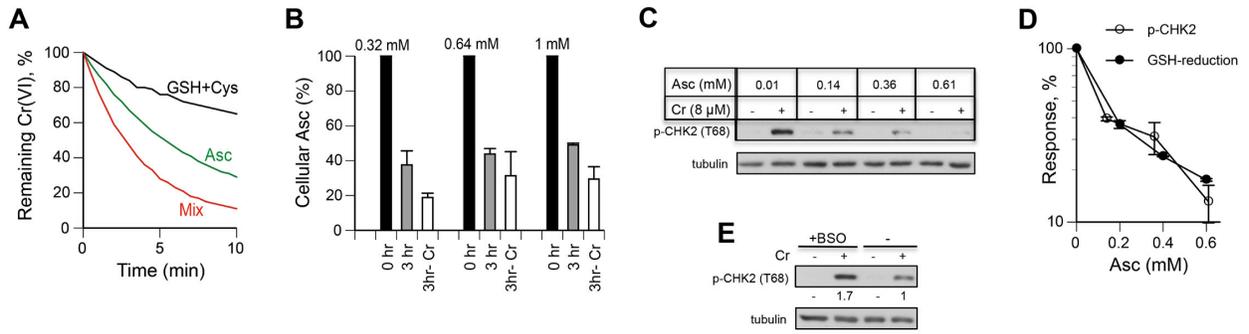


Figure 5.

