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Chromium(VI) causes interstrand DNA crosslinking *in vitro* but shows no hypersensitivity in crosslink repair-deficient human cells

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Abstract

Hexavalent chromium is a human carcinogen activated primarily by direct reduction with cellular ascorbate and to a lesser extent, by glutathione. Cr(III), the final product of Cr(VI) reduction, forms six bonds allowing intermolecular crosslinking. In this work, we investigated the ability of Cr(VI) to cause interstrand DNA crosslinks (ICLs) whose formation mechanisms and the presence in human cells are currently uncertain. We found that *in vitro* reduction of Cr(VI) with glutathione showed a sublinear production of ICLs, yield of which was less than 1% of total Cr-DNA adducts at the optimal conditions. Formation of ICLs in fast ascorbate-Cr(VI) reactions occurred during a short reduction interval and displayed a linear dose-dependence with the average yield of 1.3% of total adducts. *In vitro* production of ICLs was strongly suppressed by increasing buffer molarity, indicating inhibitory effects of ligand-Cr(III) binding on the formation of crosslinking species. The presence of ICLs in human cells was assessed from the impact of ICL repair deficiencies on Cr(VI) responses. We found that ascorbate-restored FANCD2-null and isogenic FANCD2-complemented cells showed similar cell cycle inhibition and toxicity by Cr(VI). XPA-null cells are defective in repair of Cr-DNA monoadducts but stable knockdowns of ERCC1 or XPF in these cells with extended time for the completion of crosslinking reactions did not produce any sensitization to Cr(VI). Our results together with chemical and steric considerations of Cr(III) reactivity suggest that ICL generation by chromate is probably an *in vitro* phenomenon occurring at conditions permitting formation of Cr(III) polymers.

Introduction

Compounds containing chromium in the +6 oxidation state are recognized human carcinogens based on strong epidemiological evidence for elevated risks of lung and other respiratory cancers in occupationally exposed populations.[1-3] Workers from several dozens of professional groups are known to be exposed to airborne Cr(VI). The presence of Cr(VI) in drinking water in the US and other countries is also associated with significant public health concerns.[4] Cr(VI) requires intracellular reduction to cause its genotoxic and cytotoxic effects. Ascorbate (Asc) is a principal reducer of Cr(VI) *in vivo* with small thiols glutathione (GSH) and cysteine playing a distant secondary role.[5-7] In the main target tissue of Cr(VI) toxicity, the lung, Asc is responsible for about 95% of Cr(VI) reduction.[7] In addition to its much faster reaction rate[8] and different production of oxidizing species,[9] Asc-mediated reduction of Cr(VI) at physiologically relevant concentrations of the reagents yields undetectable amounts of Cr(V)[10,11] whereas this intermediate is abundantly produced in

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GSH and Cys-based reactions.[12-14] Human and nonhepatic rodent cells in culture contain either undetectable or low-micromolar concentrations of cellular Asc,[15-17] forcing Cr(VI) into thiols-driven metabolism. Asc concentrations in tissues are typically in the range of 0.5-3 mM.[18,19] Asc deficiency of cultured cells is caused by the absence of this vitamin in the commonly used cell growth media. A standard supplementation of growth media with 10% serum can theoretically supply only 1/10th of the normal amounts of vitamin C. Asc is also irreversibly oxidized during processing and storage of serum and quickly lost from highly oxygenated, iron-rich cell culture media.

The final product of Cr(VI) metabolism by all reducers is Cr(III) that forms bulky octahedral complexes. The ability to form six bonds creates very stable complexes of Cr(III) with multidentate ligands and leads to the formation of intermolecular crosslinks (ternary adducts) with DNA *in vitro* and in cells. These include protein-Cr-DNA,[20] GSH-Cr-DNA and other crosslinks.[21] The bulkiness of the octahedral complexes with GSH and other large ligands does not preclude DNA adduction due to Cr(III) binding to the sterically accessible phosphate group.[15,22] *In vitro* reactions of Cr(VI) with Asc[23,24] and Cys[25] but not GSH[26] has also been found to produce DNA interstrand crosslinks (ICLs). Cr(III) was found to be responsible for interstrand crosslinking during Cr(VI) reduction *in vitro*.[27] ICLs are potent replication-blocking and cytotoxic lesions[28,29] and their formation is often invoked to explain cell cycle arrest by Cr(VI). However, the ability of Cr(VI) to cause ICLs in cells is currently unclear. Loss or mutations in Fanconi anemia genes cause severe hypersensitivity to DNA crosslinkers[29] and FANCA-deficient human fibroblasts were more sensitive to Cr(VI) toxicity in comparison to a normal but genetically unrelated cell line.[30] However, the same group has later found that crosslink- and nucleotide excision repair-double deficient and nucleotide excision repair-only deficient CHO mutants were equally sensitive to Cr(VI),[31] arguing against the presence of ICLs in these cells. Both studies have been performed in Asc-deficient cell cultures in which GSH dominates Cr(VI) metabolism. Since ICLs were absent in GSH reactions *in vitro*[26] but produced in Asc-driven reductions,[23,24,27] it was possible that Cr(VI) could cause ICLs in cells with physiological levels of Asc. On the other hand, the plausibility of ICL formation by Cr(VI) in cells was questioned based on the steric constrains imposed by the octahedral geometry of Cr(III).[32] Structures of Cr-induced ICLs are unknown and the linkages between N^7-dG and/or phosphate group(s) on the opposite strands have been suggested to mediate crosslinking by Cr(III).[33]

In this work, we examined DNA crosslinking by Cr(VI) *in vitro* with its two main reducers Asc and GSH and tested a potential role of ICLs in Cr(VI) toxicity in Asc-restored, isogenic human cells with different genetic defects in ICL repair.

Experimental Procedures

Caution

Cr(VI) compounds are recognized human carcinogens, and appropriate safety precautions should be taken in the handling of these materials.

Materials

L-ascorbic acid (99.9% pure), dehydro-L-(+)-ascorbic acid dimer (DHA), K_2CrO_4 (ACS reagent), glutathione (>98% pure), cisplatin [cis-diammineplatinum(II) dichloride], 4-morpholinepropanesulfonic acid (MOPS) and all salts were from Sigma (St. Louis, MO, USA). 1,2-diamino-4,5-dimethoxybenzene dihydrochloride was from Molecular Probes (Eugene, OR, USA). Mitomycin C was obtained from Tocris Bioscience (Bristol, UK). Bio-Gel P-30 chromatography columns, empty spin columns and Chelex-100 resin were purchased from Bio-Rad (Hercules, CA, USA). EcoRI restriction nuclease and DNA

polymerase I Klenow fragment were from Promega (Madison, WI, USA). The pBR322 plasmid was from New England Biolabs (Beverly, MA, USA).

Removal of trace metals

All reagents used in Cr(VI) reduction reactions were treated with Chelex-100 to remove trace amounts of iron and copper. Spin columns were packed with 0.5 mL of Chelex-100 resin and then washed with 5 volumes of 0.5 M MOPS buffer (pH 7.0) by centrifugation at room temperature in a swing-bucket rotor (2 min, 2000xg). The Chelex-containing columns were loaded with 0.5 mL of reagent stock solutions, inverted several times and then incubated for 10 min at room temperature. Purified stock solutions were eluted from the columns by gravity.

Cells and treatments

XPA-null (GM04312), FANCD2-null (GM16633) and FANCD2-complemented (GM16634) human fibroblasts were purchased from Coriell Cell Repositories (Camden, NJ, USA) and propagated in DMEM medium (12430-054 from Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum and penicillin/streptomycin. All cell lines were maintained at 37°C in 95% air/5% CO_2. Cells were treated with Cr(VI) and DNA crosslinkers for 6 hr in complete media. For assessment of cell cycle recovery from replication arrest, cells were trapped in mitosis by the addition of 0.1 μg/mL nocodazole for 18 hr after removal of test chemicals. The amount of cells progressed in mitosis in the presence of nocodazole was determined by western blotting for phospho-histone H3.

Cellular Asc

To restore Asc levels, cells were incubated for 90 min with DHA in Krebs-HEPES buffer [30 mM HEPES (pH 7.5), 130 mM NaCl, 4 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.5 mM glucose]. Stock solutions of 10 mM DHA were freshly prepared in the Krebs-HEPES buffer. Cellular concentrations of Asc were determined by measuring the amounts of its fluorescent conjugate with 1,2-diamino-4,5-dimethoxybenzene dihydrochloride.[34]

Cytotoxicity

Cell growth was assessed by the CyQUANT reagent from Invitrogen (Grand Island, NY, USA). Measurements of cell viability were based on the determination of metabolic activity using the CellTiter-Glo luminescent assay from Promega (Madison, WI, USA). Cells were seeded into 96-well plates (2000-3000 cells/well) and grown overnight prior to exposure to chemicals. For assessment of clonogenic viability, cells were seeded onto 100-mm dishes (600-1000 cells/dish), preloaded with Asc and treated with Cr(VI) for 6 hr on the next day. Colonies were fixed with methanol and stained with a Giemsa solution (Sigma) at 7-8 days post-exposure.

shRNA and retroviral infections

The pSUPER.retro.puro vector was purchased from OligoEngine (Seattle, WA, USA) and all oligonucleotides were from Integrated DNA Technologies (Coralville, IA, USA). The 60-nt long oligonucleotides containing unique 19-nt targeting sequences (5′-GTAGGATACTTGTGGTTGA-3′ for XPF and 5′-GGAGCTGGCTAAGATGTGT-3′ for ERCC1) were ligated into the linearized vector with T4 ligase overnight. Recombinant vectors were transformed into One Shot TOPO10 chemically competent *E. coli* cells (Invitrogen). Plasmid DNA was extracted from ten bacterial clones and digested with EcoRI and HindIII to confirm the presence of the insert. The vectors were co-transfected with MoMuLV gag-pol and VSVG-encoding plasmids into a packaging 293T cell line (CRL3216 from ATCC) using Lipofectamine. Virus-containing media was collected 48 hrs after

transfection, passed through 0.45 μm filters and diluted 1:1 with complete DMEM medium. XPA-null fibroblasts were infected overnight and selected with puromycin at 1 μg/mL one week post-infection.

Western blotting

Protein lysates were prepared by boiling cells in a solution containing 50 mM Tris (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol and protease/phosphatase inhibitors. Proteins were separated by SDS-PAGE and electrotransferred to ImmunoBlot PVDF membrane (Bio-Rad). Primary antibodies for Ser10-phosphorylated histone H3 (9701), histone H3 (9751) and ERCC1 (3885) were from Cell Signaling Technology, for XPF (ab17798) from Abcam, for FANCD2 (sc-20022) from Santa Cruz Biotechnology and for γ-tubulin (T6557) from Sigma.

Cr(VI) uptake

Cellular concentrations of Cr were measured by graphite furnace atomic absorption spectroscopy (GF-AAS). Cells were seeded into 6-well plates (2×10^5 cells/well) and treated with Cr(VI) the next day. After the treatments, cell monolayers were washed twice with warm PBS and cells were collected by trypsinization in the presence of EDTA. Extraction of Cr by nitric acid was performed as described previously.[35] Cr concentrations were measured using AAnalyst600 Atomic Absorption Spectrometer (Perkin-Elmer). Uptake of Cr(VI) was normalized per amount of cellular protein in each sample.

Cr(VI) reduction

Reduction of Cr(VI) was monitored by the disappearance of chromate absorbance at 372 nm. The reduction reaction was initiated by mixing equal volumes of 2× solutions of Cr(VI) and reducers. Samples were incubated at 37°C inside a microplate reader.

Quantitation of Cr-DNA adducts

DNA-bound Cr was determined by the inclusion of radiolabeled ^{51}Cr(VI) in the reaction mixtures. Reaction mixtures typically contained 25 mM MOPS (pH 7.0), 2 μg pBR322 DNA, 1 mM Asc or 10 mM GSH and Cr(VI). Samples were incubated at 37°C for 30 min (Asc reactions) or 3 hr (GSH reactions) followed by the removal of unbound components by Bio-Gel P-30 chromatography. Eluted DNA was incubated for 20 min in the presence of 200 mM NaCl and passed through P-30 columns again. ^{51}Cr radioactivity was determined using a scintillation counter.

Measurements of interstrand DNA crosslinks (ICLs)

ICLs were detected by the presence of linear plasmid molecules remaining double-stranded at highly alkali pH. pBR322 plasmids were linearized by digestion with EcoRI, purified by phenol-chloroform extraction/ethanol precipitation and then end-labeled with ^{32}P using 5 units of Klenow enzyme, 25 μM each dCTP, dTTP, dGTP and 20 μCi [α-^{32}P]dATP in a 50 μl reaction volume. The labeling reactions were incubated at 25°C for 15 min followed by the removal of unincorporated radioactivity on Bio-Gel P-30 columns. Crosslinking reactions typically contained 2 μg plasmid DNA, 25 mM MOPS buffer (pH 7.0), Cr(VI), trace amounts of ^{32}P-labeled pBR322 and Asc or GSH. After 30 min incubation at 37°C for Asc-based reactions and 3 hr for GSH reactions, unreacted Cr was removed by Bio-Gel P-30 chromatography. Eluted DNA was supplemented with 25 mM NaCl and 10 mM MOPS (pH 7.0) and then denatured by incubation with 200 mM NaOH for 10 min at room temperature. Samples were mixed with a 6x Ficoll loading buffer and immediately loaded onto 0.8% agarose gels. Radioactivity was detected by phosphoimaging. The number of ICLs per plasmid was calculated from the following equation: ICL/plasmid = -ln(1-fraction of

dsDNA). The yield of ICLs was calculated by dividing the number of ICLs per total Cr-DNA adducts.

Results and Discussion

Formation of ICLs in GSH-Cr(VI) reactions

GSH and Cys are two thiols that participate in reduction of Cr(VI) in human and animal cells *in vivo*[3]. A near complete absence of Asc in human and other cultured cells[15-17] creates conditions for the dominant role of the small thiols in Cr(VI) metabolism in cell culture models. Although Cys is a faster reducer,[8] GSH is more important for cellular reduction of Cr(VI) due to its much higher concentrations. Reduction of Cr(VI) by Cys occurs almost exclusively via one-electron transfer[37] whereas GSH reactions show a mix of one- and two-electron transfer reactions.[37] In the physiological range of GSH concentrations, one-electron reduction is predominant. The mechanistic similarity in Cr(VI) reduction would suggest that both thiols should produce similar spectra of DNA damage. However, while we have previously found that 2 mM Cys-driven reduction of Cr(VI) led to a significant formation of ICLs,[25] other investigators have been unable to detect ICL formation in Cr(VI) reactions with 0.6-12 mM GSH.[26] Since reactivity of Cr(III), the oxidative form responsible for DNA crosslinking,[27] is strongly influenced by the reaction conditions,[25] we sought to examine DNA crosslinking in GSH-Cr(VI) mixtures under the same conditions that have been previously used for Cys. We found that the majority of Cr(VI) was reduced by 10 mM GSH within 60 min incubation at 37°C (Fig. 1A). The initial rate of reduction had $t_{1/2}$=8.5 min (average of triplicate measurements). Considering potentially different late reduction processes and Cr-DNA binding, we chose 3-hr long incubations for all subsequent GSH-Cr(VI) reactions. Reduction of Cr(VI) by GSH showed essentially a linear dose-dependence in the formation of Cr-DNA adducts (Fig. 1B), pointing to a similar relative yield of DNA-reactive species at low and high Cr(VI) concentrations. Our methodology for detection of ICLs relied on the quantitation of linear dsDNA molecules that were resistant to denaturation by 200 mM NaOH. DNA unwinding by NaOH was very fast and the highly alkaline pH of this reaction blocked reactivity of Cr(III), ensuring that ICLs were not assay-generated. Figure 1C shows a complete alkali-induced unwinding of control dsDNA, resulting in the appearance of two closely moving bands of complimentary DNA strands. Reduction of increasing Cr(VI) concentrations by GSH produced a strongly sublinear formation of ICLs (Fig. 1C,D). There were no detectable ICLs at Cr(VI) concentrations below 50 μM. The maximal yield of ICLs was approximately 0.7% of total Cr-DNA adducts at the highest concentration of Cr(VI) (Fig. 1E). Thus, at our reaction conditions GSH was capable of forming ICLs although their production was relatively moderate and limited to high Cr(VI) doses.

ICLs in Asc-Cr(VI) reactions

Asc is the most important reducer of Cr(VI) *in vivo*.[5-7] We found that Cr(VI) reduction by 1 mM Asc was very rapid and more than 95% of Cr(VI) was already reduced after 5 min (Fig. 2A). The rate of reduction by 1 mM Asc ($t_{1/2}$=1 min) was approximately 8-times faster compared to 10 mM GSH (shown in Fig.1A). Because of the rapid completion of Cr(VI) metabolism, we used 30 min incubations for detection of ICLs in Asc-based reactions. Formation of total Cr-DNA adducts in Asc-Cr(VI) reactions showed a linear dose-dependence (Fig. 2B). Production of ICLs in these reactions was also near linear with respect to Cr(VI) concentrations (Fig. 2C,D). Unlike a sublinear production in GSH reactions, the yield of ICLs as a percentage of total Cr-DNA adducts was nearly constant and averaged 1.3±0.4% (Fig. 2E). The majority of ICLs were formed during reduction of Cr(VI), as the addition of DNA at 5 min after the start of reactions (>95% reduction

completion) produced only a small number of ICLs (Fig. 2F). Thus, the use of longer incubation times for our Asc reactions would not have increased the yield of crosslinks.

Cr(VI) responses in ICL repair-deficient human cells

Cr-induced ICLs catalyze DNA strand cleavage during prolonged incubations under alkaline conditions,[25] making it problematic to use of standard alkaline-based assays (filter elution, comet assay) for ICLs measurements in cells. Gene expression profiling can differentiate genotoxic from nongenotoxic chemicals but not DNA crosslinkers from other DNA damaging agents.[38] Since Cr-induced crosslinks are chemically stable at physiological conditions,[24,25] we decided to employ genetic approaches for testing the presence of toxicologically amounts of ICLs in human cells. Cells from patients with Fanconi anemia are highly susceptible to cytotoxic effects of DNA crosslinkers such as mitomycin C and cisplatin. FANCD2 plays a central role in ICL responses by integrating activity of the entire Fanconi complex consisting of several members.[28,29] Therefore, cells with mutated FANCD2 can serve as a sensitive genetic test for the formation of ICLs by Cr(VI) in cells. To validate our cellular model, we treated a pair of FANCD2-null and FANCD2-complemented human fibroblasts with mitomycin C and cisplatin and examined their growth and cytotoxicity. As expected, the lack of functional FANCD2 made cells highly sensitive to growth-inhibitory and cytotoxic effects of both DNA crosslinkers (Fig. 3A-D). The yield of ICLs by cisplatin is less than 1% of total adducts,[39] which is slightly lower than the production of ICLs in our chromate-Asc reactions (1-2%), but it was more than sufficient to observe hypersensitivity in FANCD2$^{-/-}$ cells. The yield of ICLs for mitomycin C is about 5% of total adducts,[28] which is consistent with the larger survival differences for this crosslinker in FANCD2$^{-/-}$ vs. FANCD2^{+} comparisons (Fig. 3). Estimates for ICLs production by mitomycin C are probably less accurate due to the sensitivity of its activation to biochemical factors and oxygen concentrations.

As typical for cultured cells,[15-17] both FANCD2$^{-/-}$ and FANCD2-complemented human fibroblasts had very low Asc concentrations, ranging 7-12 μM the next day after seeding. A preincubation of both cell lines with 0.5 and 1 mM DHA (dehydroascorbic acid) restored Asc concentrations to a physiological range (Fig. 4A), which is 0.5-3 mM Asc.[18,19] Asc-normalized FANCD2$^{-/-}$ and FANCD2-complemented cells showed no significant differences in accumulation of Cr(VI) (Fig. 4B). This result along with a nearly identical DHA uptake indicates the absence of any FANCD2 effect on cellular doses of Cr(VI) and its principal reducer. Using the same treatment and recovery times as in the validation studies with mitomycin C and cisplatin, we found no differences in Cr(VI) cytotoxicity between FANCD2$^{-/-}$ and FANCD2-complemented cells preloaded with 0.5 and 1 mM DHA (0.7 and 1.4 mM cellular Asc) (Fig. 4C). Cell growth inhibition and clonogenic toxicity were also unaffected by the FANCD2 status of Cr(VI)-treated cells (Fig. 4D,E). Thus, three different assays found no effect of FANCD2, indicating lack of a significant contribution of ICLs to Cr(VI) cytotoxicity in Asc-restored human cells.

FANCD2 is necessary for removal of ICLs and a subsequent restart of arrested replication forks.[29,40] Therefore, a comparison of cell cycle restoration in FANCD2$^{-/-}$ and FANCD2-complemented cells can serve as another test for the formation of ICLs by Cr(VI). A progression of Cr(VI)-treated human cells from S phase was not affected by Cr-DNA adducts,[17] which increases the sensitivity of detecting of ICLs based on replication recovery. To assess the restoration of cell cycle, we analyzed the amount of cells that were able to progress into mitosis during 18 hr recovery after DNA damage (Fig. 5A). The presence of nocodazole in these experiments trapped cells in mitosis and the size of mitotic populations was assessed from the amount of the specific biochemical marker, Ser10-phosphorylated histone H3. As expected, we found that FANCD2$^{-/-}$ cells showed a severely impaired recovery from replication arrest by mitomycin C as evidenced by the absence of phospho-

H3 at 18 hr post-treatment (Fig. 5B). Mitomycin C also strongly increased the levels of FANCD2 protein in complemented cells. Unlike mitomycin C, the ability of Cr(VI)-treated cells to progress into mitosis was not affected by their FANCD2 status based on the comparable amounts of phospho-H3 in null and complemented cells (Fig. 5C). Cr(VI) also did not induce significant changes in the levels of FANCD2 protein. Overall, our findings using the FANCD2 deficiency model do not support the formation of toxicologically significant amounts of ICLs in Asc-restored human cells.

To further explore a potential formation of ICLs in human cells, we decided to examine effects of deficiency in XPF-ERCC1 endonuclease that is important for unhooking of ICLs at the early phase of their repair.[41,42] Knockdown of XPF or ERCC1 strongly sensitized human cells to killing by ICLs.[43] XPF-ERCC1 dimer is also essential for nucleotide excision repair of Cr-DNA adducts in rodent[31] and human cells.[44] To separate defects in ICL repair and nucleotide excision repair, we depleted XPF and ERCC1 in XPA-null cells that are completely defective in nucleotide excision repair upstream of XPF activity.[45] Persistence of Cr-DNA monoadducts in XPA-null cells could also promote the formation of ICLs by providing extra time to complete the second-arm reaction by the initially formed monoadducts. We created stable knockdowns of XPF and ERCC1 proteins by retroviral infections with shRNA-encoding vectors. Figure 6A shows very efficient knockdowns of XPF and ERCC1 using our vectors. The observed co-depletion of XPF in cells expressing sh-ERCC1 was probably caused by protein destabilization in the absence of its binding partner, as it has been found for other DNA repair complexes.[46] Examination of cytotoxicity of Cr(VI) in Asc-restored XPA-null cells with depleted XPF and ERCC1 showed no significant differences from control (Fig. 6B), demonstrating the lack of toxicologically significant amounts of ICLs in the second genetic model. Consistent with our findings, XPF inactivation in hamster cells has shown no further sensitization beyond nucleotide excision repair deficiency,[31] although Asc levels in these cells were not normalized. An early study using the alkaline elution technique reported the presence of small amounts of ICLs in tissues of chromate-treated rats based on marginal, dose-independent decreases in filter elution of nuclear DNA.[47] These results could have reflected technical factors such as uneven pumping flows through filters with control and treated samples or/and incomplete digestion of DNA-protein crosslinks rather than the presence of ICLs. Cr(III) binding to peptide bonds blocks proteolysis and it is the reason for the biodurability of leather that is produced by Cr(III) binding to skin collagen.[32]

Effect of high buffer molarity on ICL formation in vitro

Cr(III) is responsible for ICL formation during reduction of Cr(VI) *in vitro*.[27] Interstrand crosslinking requires intercalation of a crosslinker between DNA bases,[28] which sterically limits crosslinking molecules to those with intercalation-permitting linear and planar geometries. The octahedral geometry of Cr(III) complexes does not allow intercalation into dsDNA, which questions the plausibility of interstrand crosslinking by this metal. It has been previously suggested that Cr(III) oligomers could cause ICLs by wrapping around the duplex and binding the opposite strands via separate Cr(III) atoms.[32] Cr(III) rapidly forms polymers at neutral pH, which is suppressed by the presence of organic ligands capable of binding Cr(III).[32] Thus, if Cr(III) polymers are important for DNA crosslinking, one would expect a diminished formation of ICLs in higher molarity buffers that can bind Cr(III) through their negatively charged sulfonate group (MES, MOPS and HEPES). We noted that a previous study reporting no ICLs in GSH reactions with 60 μM Cr(VI) used 100 mM HEPES,[26] whereas our GSH reactions with similar Cr(VI) concentrations but a lower buffer molarity (25 mM MOPS) produced detectable amounts of ICLs (Fig. 1C-E). We directly compared the formation of ICLs with different concentrations of MOPS and found that doubling molarity from 25 to 50 mM was sufficient to cause a severe suppression of DNA

crosslinking in GSH-Cr(VI) reactions (Fig. 7A). Buffer molarity had no effect on the rate of Cr(VI) reduction by GSH (Fig. 7B).

Conclusions

Reduction of Cr(VI) *in vitro* with its two main cellular reducers ascorbate and glutathione resulted in a moderate formation of DNA interstrand crosslinks, making up less than 2% of total Cr-DNA adducts at the most optimal conditions. The production of DNA crosslinks was suppressed by increasing buffer concentrations. Two types of genetic deficiencies in crosslink repair (absence of FANCD2 and XPF-ERCC1 endonuclease) produced no hypersensitivity to toxic effects of Cr(VI) in human cells with restored ascorbate levels. Our results indicate that Cr(VI) metabolism in cells does not cause toxicologically significant amounts of ICLs, whose formation occurs only *in vitro* at low buffer/ligand conditions permitting Cr(III) polymerization (Fig. 8). Intracellular abundance of Cr(III)-binding monodentate and especially multidentate ligands with negatively charged groups (amino acids, organic acids, nucleotides and others) is expected to prevent the formation of Cr(III) oligomers, which can explain the absence of ICLs in Cr(VI)-treated cells. ICLs are potent replication-blocking lesions blocking unwinding of duplex DNA,[28,29] and a normal progression from S to G2 phase in primary human lung cells treated with environmentally relevant doses of Cr(VI)[17] is also consistent with the absence of significant amounts of ICLs.

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Abbreviations

Asc	ascorbate
DHA	dehydroascorbic acid

GSH	glutathione
ICL	interstrand crosslink
MOPS	4-morpholinepropanesulfonic acid

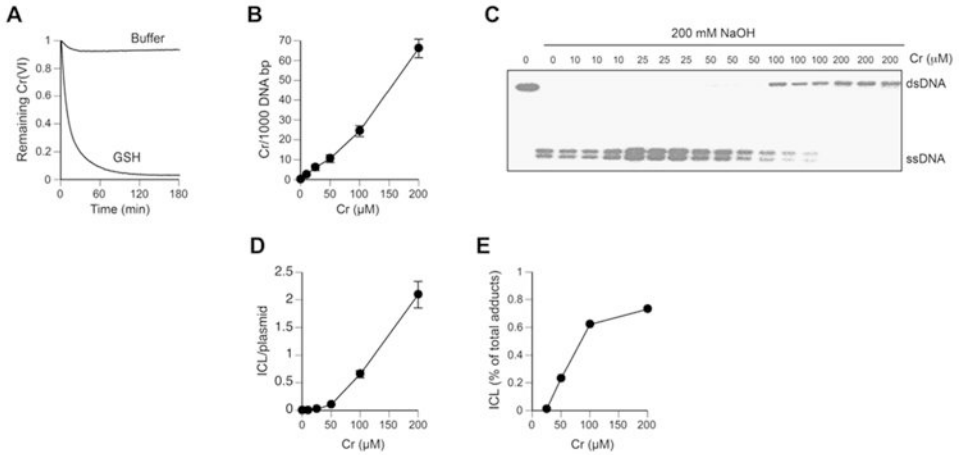

Figure 1. Formation of ICLs during reduction of Cr(VI) with GSH

Reaction mixtures contained 10 mM GSH, 25 mM MOPS (pH 7.0), 2 μg DNA and indicated concentrations of potassium chromate. All reduction reactions were incubated for 3 hr at 37°C. Data are means+SD (n=3-4). Where not seen, error bars are smaller than data symbols. **(A)** Reduction of 100 μM Cr(VI) by GSH. **(B)** Formation of Cr-DNA adducts in Cr(VI)-GSH reactions. **(C)** Representative radiogram demonstrating the presence of dsDNA in Cr(VI)-treated samples after denaturation with 200 mM NaOH. **(D)** Dose-dependence of ICL formation by Cr(VI). **(E)** Yield of ICLs as a percentage of total Cr-DNA adducts.

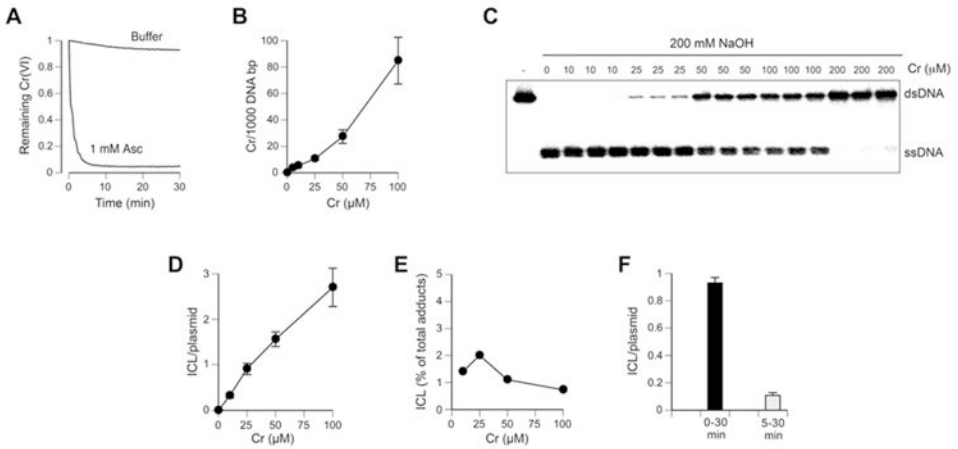

Figure 2. Formation of ICLs during reduction of Cr(VI) with Asc

Reaction mixtures contained 1 mM Asc, 25 mM MOPS (pH 7.0), 2 μg DNA and indicated concentrations of Cr(VI). All reduction reactions except for data in panel F were incubated for 30 min at 37°C. Data are means±SD (n=3-5). Where not seen, error bars are smaller than data symbols. **(A)** Reduction of 100 μM Cr(VI) by Asc. **(B)** Formation of Cr-DNA adducts in Cr(VI)-Asc reactions. **(C)** Representative radiogram demonstrating the presence of dsDNA in Cr(VI)-treated samples after denaturation with 200 mM NaOH. **(D)** Dose-dependence of ICL formation by Cr(VI). **(E)** Yield of ICLs as a percentage of total Cr-DNA adducts. **(F)** Formation of ICLs during Cr(VI) reduction (0-30 min samples) and after the completion of Cr(VI) reduction (5-30 min samples). All reactions contained 50 μM Cr(VI).

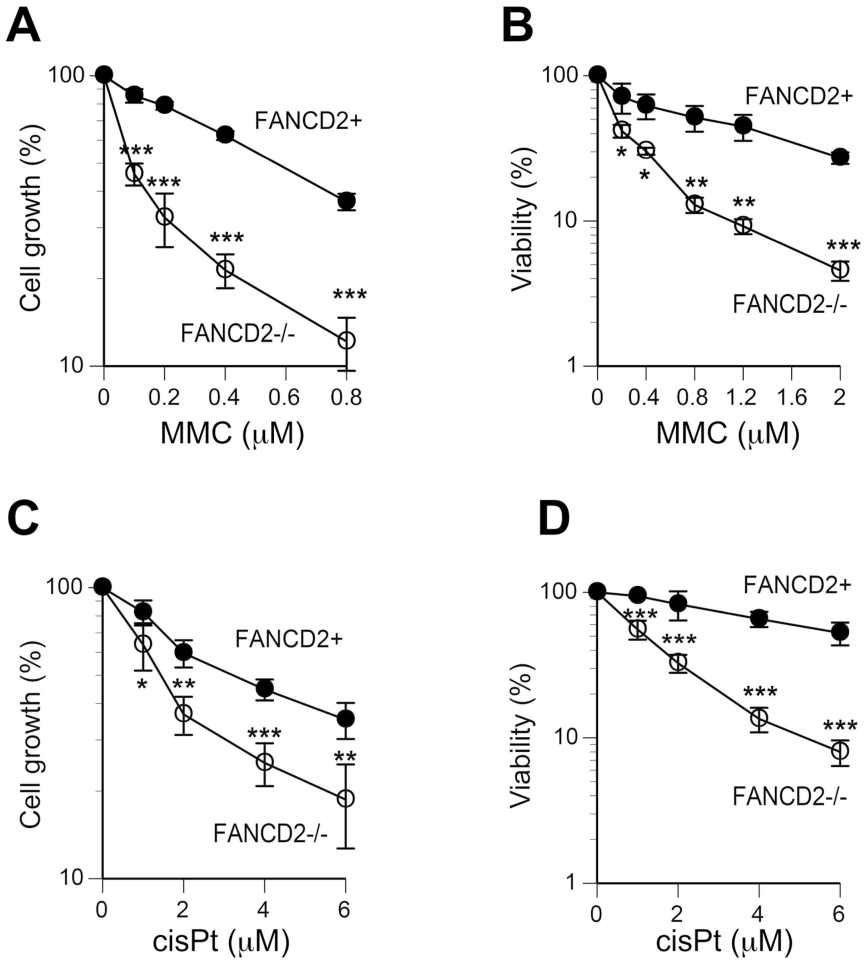

Figure 3. Hypersensitivity of FANCD2-null human fibroblasts to cisplatin and mitomycin C

Isogenic FANCD2-null (FANCD2-/-) and FANCD2-complemented (FANCD2+) cells were treated with mitomycin C and cisplatin for 6 hr in complete media and cytotoxicity measurements were taken 72 hr later. Data are means±SD, n=4, *-$p<0.05$, **- $p<0.01$, ***-$p<0.001$. **(A)** Cell growth and **(B)** Viability of mitomycin C (MMC)-treated FANCD2-/- and FANCD2+ fibroblasts. **(C)** Cell growth and **(D)** Viability of cisplatin (cisPt)-treated FANCD2-/-and FANCD2+ fibroblasts.

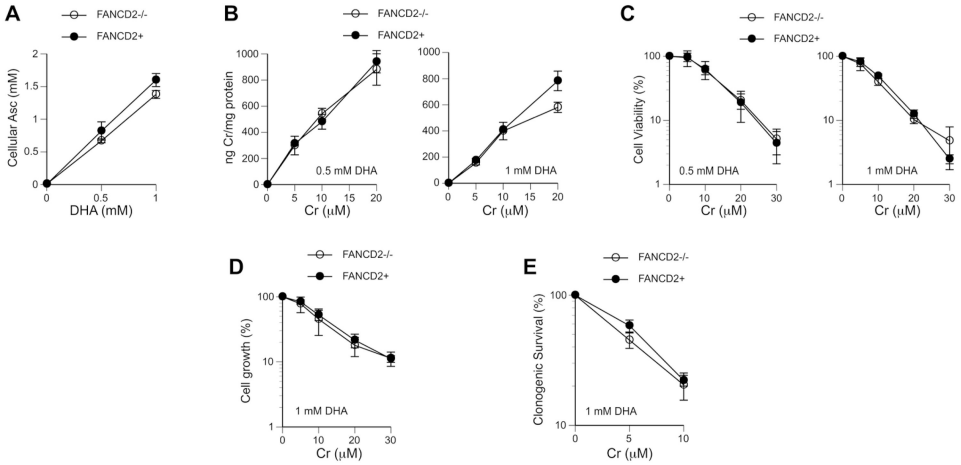

Figure 4. Uptake and cytotoxicity of Cr(VI) in FANCD2-null and FANCD2-complemented human fibroblasts

Data are means±SD (n=3-6). Cells were preincubated with the indicated concentrations of DHA for 90 min prior to the addition of Cr(VI). All treatments with Cr(VI) were 6-hr long. **(A)** Cellular concentrations of Asc after preincubation with DHA. **(B)** Cr(VI) uptake by cells preincubated with 0.5 and 1 mM DHA. **(C)** Cell viability at 72 hr after exposure to Cr(VI). **(D)** Growth inhibition by Cr(VI). **(E)** Clonogenic survival of Cr(VI)-treated cells.

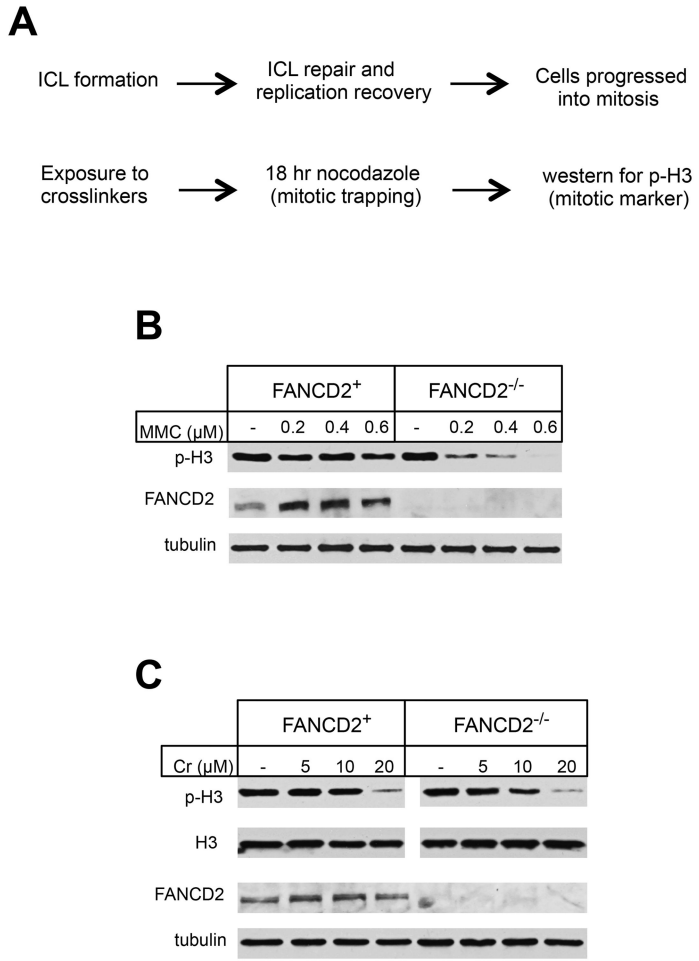

Figure 5. Importance of FANCD2 for recovery of cell cycle after exposure to mitomycin C but not Cr(VI)

(A) A flow chart describing an experimental approach for testing the ability of cells to recover from replication arrest and progress into mitosis. **(B)** A severe defect in the ability of FANCD2-null cells to recover from mitomycin C (MMC)-induced replication arrest. Cells were trapped in mitosis by the addition of 0.1 μg/mL nocodazole for 18 hr after removal of MMC. **(C)** A normal progression of FANCD2-null cells into mitosis after Cr(VI) exposure. Cells were trapped in mitosis by the addition of 0.1 μg/mL nocodazole for 18 hr after Cr(VI) exposure.

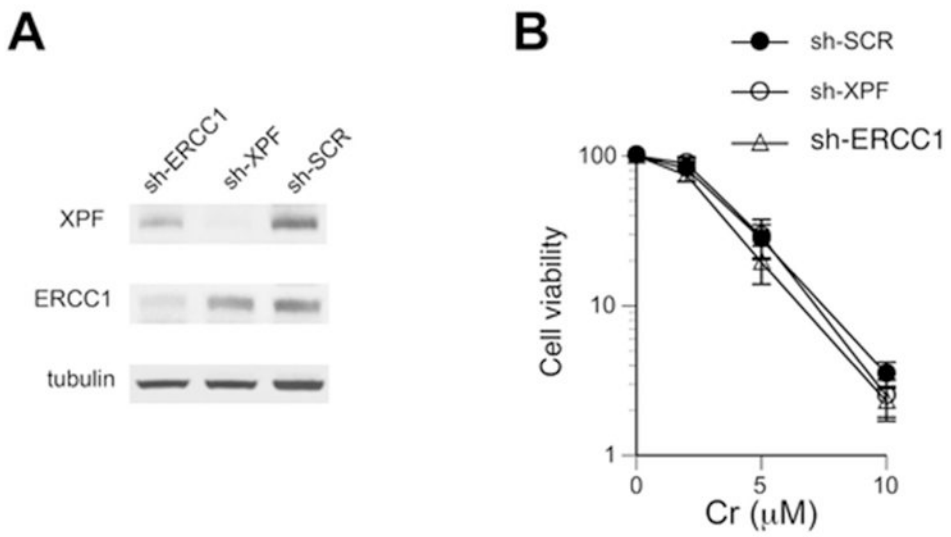

Figure 6. Cytotoxicity of Cr(VI) in cells with shRNA-depleted XPF-ERCC1 endonuclease

(A) Western blots demonstrating XPF and ERCC1 knockdowns in XPA-null human fibroblasts. **(B)** Cytotoxicity of Cr(VI) in control (shSCR) and XPF/ERCC1-depleted (shXPF/shERCC1) cells. Cells were preloaded with 2.5 mM Asc prior to 6-hr long treatment with Cr(VI) and cell viability was measured at 72 hr post-exposure. Data are means±SD (n=6).

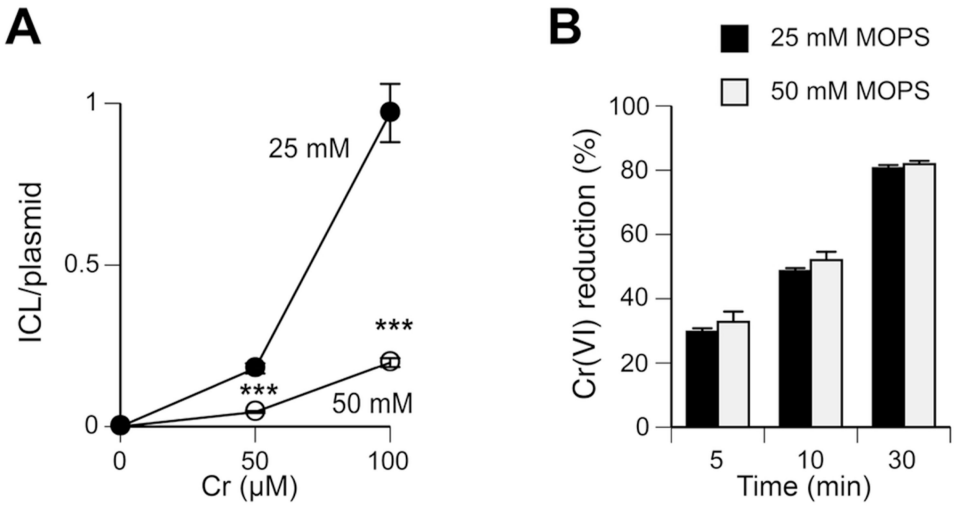

Figure 7. Suppression of ICL formation by increasing buffer molarity

Reaction mixtures contained 10 mM GSH, MOPS buffer (pH 7.0), 2 μ*g* DNA, and 0-200 μM Cr(VI) and were incubated for 3 hr at 37°C. Data are means±SD (n=3, ***-p<0.001). Where not seen, error bars are smaller than data symbols. **(A)** ICL formation in Cr(VI)-GSH reactions containing 25 or 50 mM MOPS. **(B)** Reduction of Cr(VI) by 10 mM GSH in the presence of 25 and 50 mM MOPS (pH 7.0).

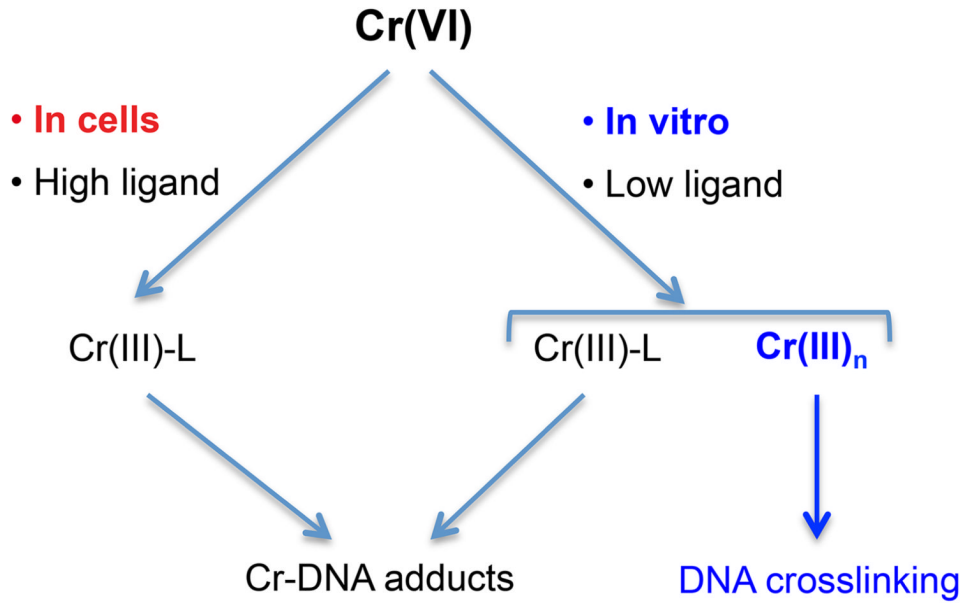

Figure 8. A flow chart depicting Cr(VI) reactions leading to the formation of DNA monoadducts and DNA interstrand crosslinks

L - any buffer or other ligand capable of moderate or strong binding to Cr(III), $Cr(III)_n$ – polymeric Cr(III) products.