Cyclosporin A inhibits chromium(VI)-induced apoptosis and mitochondrial cytochrome c release and restores clonogenic survival in CHO cells

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Introduction

The apoptotic signaling pathway consists of a cascade of molecular events which lead from the initiation of cell death to eventual dismantling of the cell. The point at which the cell is irrevocably committed to death is unclear. Inhibitors of the proteolytic caspase cascade are able to block some or all of the morphological consequences associated with apoptosis, but they appear to be unable to maintain clonogenic or long-term survival (1–3). Cells exposed to pro-apoptotic stimuli in the presence of caspase inhibitors will either undergo terminal growth arrest or will die by some slower non-apoptotic mechanism of cell death (4). Upstream of the caspases, a variety of key events in apoptosis occur which involve the mitochondria, including loss of mitochondrial transmembrane potential, activation of the mitochondrial permeability transition (MPT), the participation of bcl-2 family proteins and the release of caspase activators. If a mechanism for commitment to cell death exists, it may involve these mitochondrial events.

Cytochrome c (cyt c) is released from the mitochondrial intermembrane space to the cytosol where it forms a complex with Apaf-1 and caspase-9, thereby triggering caspase-9 activation and initiating the caspase cascade (5–7). It has been suggested that cyt c release may occur in response to a collapse of the mitochondrial inner transmembrane potential (3). The loss of membrane potential indicates a MPT caused by opening of a large conductance MPT pore (8). Studies with isolated mitochondria show that the MPT pore favors a closed state, but certain physiological and pathological signals trigger pore opening (9–11). The fully opened state creates a channel for cyt c release without concomitant organelar swelling. Cyclosporin A (CsA), an immunosuppressant, can inhibit the MPT. CsA binds to Cyp-M, a cyclophilin-family protein associated with the MPT pore, preventing cyt c release and did not increase clonogenic survival of Cr(VI)-treated CHO cells. In contrast, the general caspase inhibitor Z-VAD-FMK markedly inhibited most of the morphological and biochemical parameters of apoptosis but did not prevent cyt c release and did not increase clonogenic survival. These results suggest that the MPT plays an important role in the regulation of mitochondrial cyt c release and that this may be a critical point in the apoptotic pathway in which cells are irreversibly committed to death.

Abbreviations: csA, cyclosporin A; cyt c, cytochrome c; DMSO, dimethyl sulfoxide; IDF, internucleosomal DNA fragmentation; MPT, mitochondrial permeability transition; PBS, phosphate-buffered saline; Z-VAD-FMK, Z-Val-Ala-Asp(OMe)-FMK.

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response to Cr(VI) in Chinese hamster ovary (CHO) cells in culture (30,31). Therefore, we used CHO cells exposed to Na₂CrO₄ as a model for apoptotic induction caused by diverse genotoxic exposure.

In this study we show that Cr(VI) exposure causes a dose-dependent release of cyt c and we examine the effect of csA on cyt c release, apoptosis and clonogenic survival in Cr(VI)-exposed CHO cells. csA affects the apoptotic signaling pathway upstream of the caspase cascade, therefore, we investigated the difference in its effects compared with the broad spectrum caspase inhibitor Z-VAD-FMK. The ability of csA to inhibit cyt c release and increase clonogenic survival, whereas Z-VAD-FMK does not, suggests that apoptotic events that occur at the mitochondria may denote a `point of no return' in the cell death pathway. Furthermore, cells that are rescued from Cr-induced apoptosis by treatment with csA would survive despite the genotoxic effects of Cr. This would potentially promote the survival of genetically damaged cells which may contribute to Cr(VI)-induced carcinogenesis.

Materials and methods

Cell culture

CHO A8 cells were maintained in Eagle’s basal medium containing 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies, Gaithersburg, MD) and 2 mM glutamine (Life Technologies) in a 95% air/5% CO₂ humidified atmosphere at 37°C.

Treatment of cells with chromium, Z-VAD-FMK and csA

Sodium chromate (Na₂CrO₄·4H₂O) (J.T. Baker Chemical Co., Phillipsburg, NJ) was dissolved in double distilled H₂O and sterilized through a 0.2 µm filter before use. Cells were treated with a final concentration of 0.3, 3, 6, or 12 µM Na₂CrO₄ for 24 h in complete medium. After 24 h the medium was replaced and the cells were incubated for an additional 24 h period. csA (Sigma, St Louis, MO) was dissolved in dimethyl sulfoxide (DMSO). Cells were pre-treated for 30 min prior to the Na₂CrO₄ exposure and co-treated for the 24 h Na₂CrO₄ exposure period with a final concentration of 5 µM csA. Z-Val-Ala-Asp(Ome)-FMK (Z-VAD-FMK) (Enzyme Systems Products, Livermore, CA) was dissolved in DMSO and added to cells immediately prior to the 24 h Na₂CrO₄ exposure period at a final concentration of 50 µM. The final concentration of DMSO in the medium never exceeded 0.1%. Control samples were exposed to an equivalent concentration of DMSO.

Interunucleosomal DNA fragmentation (IDF) analysis

IDF was analyzed after the 24 h post-Na₂CrO₄ exposure period. Cells were collected by trypsinization and combined with non-adherent cells from the culture medium. The cells were centrifuged at 600 × g for 5 min. Cell pellets were washed once in phosphate-buffered saline (PBS) and lysed in 500 µL of digestion buffer (100 mM Tris–HCl, pH 8.0, 200 mM NaCl, 10 mM EDTA, 4% SDS and 0.2 mg/mL proteinase K), followed by incubation at 55°C for 18 h. DNA was extracted with phenol/chloroform and then chloroform alone, incubated with 0.1 mg/mL RNase A for 30 min at 37°C and finally precipitated with 100% ethanol. Sample volumes equivalent to 7.5 µg were electrophoresed on a 1% agarose gel with 0.5 µg/mL ethidium bromide and visualized with an Eagle Eye II still video imaging system (Stratagene, La Jolla, CA).

Preparation of cytosolic fractions

Cr-treated cells were harvested by gentle cell scraping and combined with non-adherent cells from the culture medium. The cells were centrifuged at 300 × g for 5 min at 4°C and washed once with ice-cold PBS. Cell pellets were resuspended at 1.0–2.0×10⁵ cells/ml in ice-cold isotonic cytosol buffer (210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.5, 0.2 mM EDTA, 1 mM MgCl₂, 5 mM glutamic acid, 5 mM malate, 0.1 mM phenylmethylsulfonyl fluoride, supplemented with protease inhibitors (5 µg/mL pepstatin A, 10 µg/mL leupeptin, 2 µg/mL aprotonin) (Sigma). Cell suspensions were stirred for 10 min and then centrifuged at 12,000 × g for 15 min at 4°C to remove cell membrane debris, mitochondria and other organelles. The resulting supernatants were concentrated through Millipore Ultrafree-CL filters and stored at −20°C until used for PAGE and western blot analysis.

For measurement of the full cyt c content of a cellular sample, cytoplasmic extracts were isolated in a hypotonic solution. This cell fraction contains lysed mitochondria and pieces of plasma membrane and other organelles, but not whole cells or nuclei. Cell pellets were resuspended at 1.0–2.0×10⁵ cells/ml in ice-cold hypotonic cytosol buffer (20 mM KCl, 20 mM HEPES, pH 7.5, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 mM CaCl₂, supplemented with protease inhibitors (5 µg/mL pepstatin A, 10 µg/mL leupeptin, 2 µg/mL aprotonin) (Sigma). Cell suspensions were vortexed for 10 min and then centrifuged at 5000 × g for 15 min at 4°C. The resulting supernatants were concentrated through Millipore Ultrafree-CL filters and stored at −20°C until used for analysis.

Protein electrophoresis and western blotting

Protein concentrations of the cytosolic fractions were determined with a DC Protein Assay Kit II (Bio-Rad, Hercules, CA). Samples containing an equal amount of concentrated proteins were separated on a 4–20% gradient SDS–polyacrylamide gel and transferred to a polyvinylidene difluoride membrane by electroblotting for 1 h at 30 V at 4°C. Non-specific membrane binding sites were blocked overnight at 4°C with blocking solution (TBS-T (2.7 mM KCl, 138 mM NaCl, 20 mM Tris base, pH 7.4, 0.1% Tween-20) containing 5% non-fat dry milk). The membrane was incubated with primary mouse anti-cyt c monoclonal antibody (Pharmingen, San Diego, CA) diluted 1:2000 in blocking solution (0.5 µg/ml) for 1 h at room temperature. The membrane was washed thoroughly with TBS-T and then incubated for 1 h with a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Amersham, Arlington Heights, IL) diluted 1:2000 in blocking solution. The secondary probe was detected by using the Renaissance enhanced chemiluminescence detection system (NEN, Boston, MA).

Results

To determine if Cr(VI)-induced apoptosis involves mitochondrial disruption, we isolated the cytosolic fractions of apoptotic CHO cells and analyzed them for the presence of cyt c. We based our Cr(VI) concentrations on previously reported dose-dependent increases in CHO cell apoptosis and decreases in clonogenicity following Cr(VI) exposure (32). We isolated the cytosolic fractions using a method based on selective plasma membrane permeabilization in isotonic buffer containing low concentrations of digitonin (33,34). This method allows isolation of cyt c without contamination with mitochondria-associated cyt c. We carefully titrated the level of digitonin used in the isotonic buffer to determine the optimal conditions for plasma membrane permeability without mitochondrial membrane permeability in CHO cells. We observed that a 24 h exposure to different concentrations of Na₂CrO₄ caused a dose-dependent increase in cytosolic levels of cyt c (Figure 1A). Densitometric analysis of independent western
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**Fig. 1.** Cr(VI) causes dose-dependent release of cyt c. (A) Cytosolic fractions of CHO cells exposed to different concentrations of Na$_2$CrO$_4$ were collected and analyzed for cyt c by western blotting. The Na$_2$CrO$_4$ doses ranged from 0 to 12 µM as indicated. Purified bovine heart cyt c was used as a standard (St). (B) Densitometric analysis of cyt c western blots shows that the relative density of cytosolic cyt c increases significantly at 6, 9 and 12 µM Na$_2$CrO$_4$. Results are the means ± SE of three independent experiments. The * denotes the sample mean of two independent experiments.

Blots showed that at the 3 µM dose only a minor increase in cytosolic cyt c was observed (Figure 1B). However, the cytosolic cyt c levels increased as the Na$_2$CrO$_4$ dose increased from 3 to 12 µM. The increases in cytosolic cyt c levels were statistically significant by Student’s t-test at the 6, 9 and 12 µM Na$_2$CrO$_4$ doses ($P = 0.024$, $2.3 \times 10^{-7}$ and $7.4 \times 10^{-4}$, respectively). Cyt c release was maximal at the highest dose tested (12 µM), which corresponds to nearly 100% apoptotic cell death in CHO cells (data not shown). Cytosolic samples with the full mitochondrial cyt c content isolated using a hypotonic buffer were also analyzed. The level of cyt c release at the 12 µM dose was ~75% of the total cyt c content of the hypotonic control sample (data not shown).

Cytosolic fractions isolated from cells exposed to 9 µM Na$_2$CrO$_4$ and pre-treated for 30 min and co-treated for 24 h with 5 µM CsA were analyzed for the presence of cyt c by western blotting. Treatment with CsA markedly inhibited the cyt c release induced by Na$_2$CrO$_4$ (Figure 2A). In contrast, the general caspase inhibitor Z-VAD-FMK had no effect on the release of cyt c (Figure 2B). Densitometric analysis of independent western blots showed that only 24% of the cyt c release induced by 9 µM Na$_2$CrO$_4$ exposure was observed when cells were treated with CsA as compared with cells treated with Z-VAD-FMK or solvent alone (Figure 2C).

Degradation of DNA into 180–200 bp oligonucleosomal fragments was evident in CHO cells treated with 9 µM Na$_2$CrO$_4$ and co-treated with either CsA or Z-VAD-FMK compared with the samples which received solvent only (Figure 2C).

**Fig. 2.** Inhibition of Cr(VI)-induced mitochondrial cyt c release by CsA but not by Z-VAD-FMK. (A) A 30 min pre-treatment and 24 h co-treatment with 5 µM CsA was given to CHO cells exposed to 0 or 9 µM Na$_2$CrO$_4$. Cytosolic fractions of these cells were compared with cytosols of cells treated with CsA as compared with cells treated with solvent or Z-VAD-FMK alone (St) in the first lane. (B) CHO cells exposed to 0 or 9 µM Na$_2$CrO$_4$ were either treated with 50 µM Z-VAD-FMK (Z-VAD) or a solvent control and the cytosols were compared for the presence of cyt c by western blot analysis. An aliquot of 10 ng of purified bovine heart cyt c was used as a standard (St) in the first lane. (C) Densitometric analysis of independent western blots shows the percentage of cytosolic cyt c in samples treated with 9 µM Na$_2$CrO$_4$ and co-treated with either CsA or Z-VAD-FMK compared with the samples which received solvent only. Results are the means ± SE of three independent experiments.
Increased clonogenicity of Cr-exposed CHO cells by csA but not by Z-V AD-FMK. CHO cells exposed to increasing concentrations of Na$_2$CrO$_4$ for 24 h, and either treated with 5 µM csA, 50 µM Z-V AD-FMK or a solvent control were analyzed for cloning efficiency. The number of colonies for the indicated concentrations of Na$_2$CrO$_4$ are expressed as a percentage of the 0 µM control for each group. Results are the means ± SE of three independent experiments.

Fig. 3. Reduction of Cr-induced apoptosis by csA and Z-V-AD-FMK. (A) Genomic DNA was isolated from CHO cells exposed to 0 or 9 µM Na$_2$CrO$_4$ for 24 h, and either treated with 5 µM csA, 50 µM Z-V-AD-FMK or a solvent control. The DNA was isolated 24 h after exposure and analyzed for IDF. A 1 kb ladder was used as a DNA marker in lane 1. (B) Densitometric analysis of IDF shows the percentage of cytosolic cyt c from samples treated with 9 µM Na$_2$CrO$_4$ and co-treated with either csA or Z-V AD-FMK compared with the samples which received solvent only. Results are the means ± SE of three independent experiments.

Fig. 4. Increased clonogenicity of Cr-exposed CHO cells by csA but not by Z-VAD-FMK. CHO cells exposed to increasing concentrations of Na$_2$CrO$_4$ for 24 h, and either treated with 5 µM csA, 50 µM Z-VAD-FMK or a solvent control were analyzed for cloning efficiency. The number of colonies for the indicated concentrations of Na$_2$CrO$_4$ are expressed as a percentage of the 0 µM control for each group. Results are the means ± SE of three independent experiments.

induced IDF, verifying the dependence for IDF on caspase activity. Treatment with 5 µM csA was also able to reduce IDF induced by 9 µM Na$_2$CrO$_4$, indicating that cyt c release may also be a necessary component of the IDF pathway. Densitometric analysis of three independent IDF gels showed that Z-VAD-FMK is able to reduce the amount of IDF to 36% of that caused by 9 µM Na$_2$CrO$_4$ alone, while csA can reduce Cr-induced IDF to a comparable 41% (Figure 3B).

Since csA was shown to block mitochondrial cyt c release and reduce apoptosis, we examined whether it could restore clonogenic survival to Cr-treated CHO cells. Clonogenicity is an indicator of a cell’s long-term survival and ability to grow and form colonies in culture after exposure to a toxic agent. Clonogenicity reflects both apoptosis and long-term growth arrest caused by the toxic exposure. The clonogenicity of CHO cells exposed to different concentrations of Na$_2$CrO$_4$ alone and with pre- and co-treatments with csA or Z-VAD-FMK was determined (Figure 4). At the 3 and 6 µM doses csA was able to increase the number of colonies that formed. The increase in clonogenicity was statistically significant by Student’s t-test at the 3 and 6 µM Na$_2$CrO$_4$ doses (P = 0.021 and 0.003, respectively). Exposure to 3 µM Na$_2$CrO$_4$ decreased clonogenic survival to 57% compared with the 0 µM control. Pre- and co-treatment with 5 µM csA at 3 µM Na$_2$CrO$_4$ increased the clonogenicity to nearly 100% of control. The 6 µM dose reduced clonogenicity to 9% of control, but csA increased this clonogenic potential to 30%. We observed the same trend at the highly toxic 9 µM dose, although it was not statistically significant. In contrast, Z-VAD-FMK had no effect on clonogenicity of Cr-exposed CHO cells.

Because csA, but not Z-VAD-FMK, increased clonogenic survival after chromium treatment, we sought to determine whether csA affected uptake of Cr(VI) from the medium.
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Exposure to apoptotic doses of Cr(VI) causes mitochondrial instability, as indicated by the release of cyt c into the cytosol (Figure 1). It is believed that cyt c release is necessary for activation of the caspase cascade or, in some cases, at least for the amplification of caspase activity required for a normal apoptotic response (6,7,12). Here we show that treatment with the caspase inhibitor Z-VADE-FMK is able to inhibit DNA fragmentation associated with apoptosis (Figure 3) but does not affect long-term clonogenic survival (Figure 4). Despite inhibition of the morphological apoptotic characteristics caused by active caspases, the cells never regain replicative competency and eventually die. The point at which a cell is committed to death should, therefore, occur upstream of the caspase cascade.

CsA functions at the mitochondria by promoting mitochondrial stability and preventing the release of caspase activators. It is believed to bind at the MPT pore, thereby increasing the probability of pore closure and preventing disruption of the mitochondrial inner membrane potential (13,14). Our results indicate that treatment with CsA is able to inhibit apoptosis (Figure 3) and block mitochondrial cyt c release (Figure 2). Most importantly, CsA treatment is able to restore long-term clonogenic survival to cells that have been exposed to apoptotic doses of Cr(VI) (Figure 4). These results suggest that maintaining mitochondrial stability may allow cells to escape apoptotic death and that the point along the apoptotic pathway in which cells are committed to death involves mitochondria. It is unclear whether the ‘point of no return’ in cell death is disruption of the mitochondrial membrane potential or release of mitochondrial caspase activators. These two events may be intricately linked, however, some studies have shown that the release of cyt c can occur before or in the absence of disruption of the transmembrane potential (37,38). It is possible that different regulatory events which control mitochondrial membrane permeability are dependent on cell type. Nevertheless, it seems likely that uncoupling of the respiratory chain and subsequent release of cyt c from the mitochondria would prevent long-term cell survival. Release of cyt c from a mitochondrion should cause an irreversible disruption of oxidative phosphorylation, rendering the mitochondrion non-functional for energy metabolism and cell growth. The extent of cyt c release may vary for each cell depending on the individual molecular and cellular factors that govern apoptosis. Thus it is possible that when a critical amount of cyt c is released from mitochondria the cell is committed to death, either apoptotic or otherwise.

At lower doses (3 µM) of Cr(VI) only a small amount of cyt c release (reproducible but not statistically significant) could be observed at one particular time point (Figure 1). Interestingly, these low doses caused a significant decrease in clonogenic survival (57% of control) over a longer course of time. Cyt c release at lower doses of Cr(VI) may occur gradually followed by a protracted increase in apoptosis. Therefore, this response accounts for the significant decrease in clonogenic survival, but a marked release of cyt c at any one moment in time would not be expected. Nevertheless, treatment with CsA was able to significantly increase the long-term survival of the cells, raising clonogenicity from 57 to nearly 100% (Figure 4). CsA was not able to significantly increase the clonogenic survival of cells treated with the highest dose of Cr(VI) (9 µM). This is undoubtedly due to the presence of an insurmountable amount of damage to critical

Importantly, CsA did not alter Cr uptake by CHO cells (Figure 5). CHO cells treated with CsA and exposed to different levels of Na₂⁵¹CrO₄ for 3 h had similar levels of intracellular ⁵¹Cr to the solvent controls.

Discussion

Dysregulation of the homeostasis between cell proliferation and cell death is involved in a vast array of human health consequences, including tumorigenesis. If apoptosis fails to occur normally when cells have been damaged or injured, then it may contribute to the progression of cancer. In studying the relationship between apoptosis and cancer, genotoxic carcinogens are particularly interesting because in order to induce neoplastic transformation, doses that induce a certain level of apoptosis are often required (16). In the case of Cr(VI), exposure to levels of particulate Cr compounds that are associated with lung cancer usually also cause respiratory toxicity that manifests as perforation of the nasal septum and/or respiratory tract ulcerations (16). These conditions are associated with high levels of cell death, thus a key question is how and why do certain cells exposed to a carcinogen escape death when others die? In tissue exposed to a Cr(VI) compound, individual cells may experience different local concentrations that are possibly genotoxic but not apoptotic. The populations of cells which either escape or are resistant to apoptosis may become the precursor pools out of which neoplastic variants will emerge. Therefore, it is important to determine the point in the apoptotic pathway when a cell is irrevocably committed to death. Our results here suggest that the ‘point of no return’ for chromium-induced apoptosis occurs at the mitochondria and that cells that survive an apoptotic challenge may have a selective advantage in their ability to maintain mitochondrial stability.
macromolecules making it impossible for the cells to regain replicative competence even if apoptosis is prevented.

Here we have shown that the anti-apoptotic and pro-survival effects of csA are not the result of altered cellular uptake of Cr(VI) (Figure 5). We have previously shown that treatment of cells with Cr(VI) causes structural DNA damage in the form of adducts, single-strand breaks, DNA crosslinks and chromosomal abnormalities (39). Some of this structural damage leads to functional damage in the form of base-specific DNA and RNA polymerase arrest and inhibition of DNA replication and transcription. The cellular response to this damage includes altered gene expression, G1/S and S phase cell cycle arrest, terminal growth arrest and apoptosis (21,23,28–31). Survivors exhibit DNA deletion mutations and may progress to neoplastic transformation (39). We have observed that p53 levels are increased in Cr(VI)-treated cells (40) and, presumably, this increase would be part of the cellular response to genotoxicity, contributing to G1/S checkpoint arrest and apoptosis in the presence of excessive unreparable damage. CHO cells express a mutant p53 which alters the G1/S checkpoint arrest arm of the p53 molecular response pathway (41,42), but they still undergo apoptosis after Cr(VI) treatment.

Nevertheless, the p53 status of the cell line does not negate the observation that csA inhibited apoptosis and increased clonogenic survival, whereas Z-VAD-FMK inhibited apoptosis but had no effect on clonogenic survival. Experiments on p53 wild-type diploid human lung cells are in progress but are difficult and slow due to the nature of the primary cell cultures. The ability of cells to undergo apoptosis in response to genotoxic insult may represent a protective mechanism against neoplastic transformation in the organism by eliminating cells that contain unrepaired genetic lesions. The cells that survive genotoxic damage by escaping apoptosis and gradually regaining replicative competence may harbor the deleterious effects of the apoptotic insult that induced death in the rest of the cell population. Thus, these cells may be predisposed to further progression towards a neoplastic phenotype. CsA, widely used as an immunosuppressive drug to treat autoimmune diseases and prevent rejection in organ transplantation, has many common side-effects, including an increased risk of cancer (43). The ability of CsA to reduce apoptosis may be a consideration as a mechanism for the increased risk of cancer in patients who take CsA long term. In this study, cells acquired resistance to apoptosis by interference with the mitochondrial instability conveyed by the pharmacological treatment. However, this raises the interesting question of whether the cells that survive apoptotic doses of a chemical carcinogen such as Cr(VI), in the absence of any pharmacological intervention, have a stochastic resistance to mitochondrial instability. By way of this resistance, such a sub-population of cells may be selected for by the apoptotic inducer, thereby increasing the population of cells resistant to apoptosis. If early tumor growth requires a net accumulation of cells due to alterations in the growth/death ratio, then selection of cells with resistance to apoptosis may facilitate the early steps of carcinogenesis.

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