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Molecular mechanisms of Cr(VI)-induced carcinogenesis

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Abstract

Although Cr(VI)-containing compounds are well documented carcinogens, their mechanism of action is still not well understood. Recent studies have suggested that reduction of Cr(VI) to its lower oxidation states and related free radical reactions play an important role in Cr(VI)-induced carcinogenesis. This article summarizes recent studies from our laboratory on (a) the reduction of Cr(VI) by ascorbate, diol- and thiol-containing molecules, certain flavoenzymes, cell organelles, intact cells, and whole animals; (b) free radical production in both non-cellular and cellular systems; and (c) Cr(VI)-induced DNA damage, activation of nuclear transcription factor κB (NF-κB), activator protein-1, p53, hypoxia-inducible factor-1, vascular endothelial growth factor, tyrosine phosphorylation, apoptosis, cell growth arrest, and gene expression profile. (Mol Cell Biochem **234/235**: 293–300, 2002)

Key words: Cr(VI), carcinogenesis, reactive oxygen species, signal transduction, transcriptional regulation, cell growth arrest, apoptosis

Introduction

Chromate (Cr(VI)) compounds, widely used in industry, have been shown to have serious toxic and carcinogenic effects on humans [1–4]. Epidemological studies among industrially exposed chromium workers have identified chrome plating, chrome pigment, leather tanning, and stainless steel production as sources of potential exposure to this metal [1]. Because of its wide industrial use, environmental contamination is an additional source of human exposure to this metal. Cr(VI) has been shown to induce chromosomal aberrations, mutations, and transformations in cultured mammalian cells [5–7], and a variety of DNA lesions such as strand breaks, DNA protein cross-links and DNA base modification [8–11]. In contrast, most Cr(III) compounds are relatively non-toxic, non-carcinogenic and non-mutagenic [6]. In a number of studies [12, 13], it has been shown that Cr(VI) readily enters cells while Cr(III) does not. A nonspecific anion channel is the common mechanism for Cr(VI) uptake in exposed hosts. Being predominantly octahedral, Cr(III) ions can cross the membranes only very slowly via simple diffusion or pinocytosis. Since Cr(VI) itself does not react readily with isolated DNA [14], the reduction of Cr(VI) by cellular reductants to its lower oxidation states, Cr(V), Cr(IV) and Cr(III), has been considered an important step [15]. While Cr(V) intermediates have been reported to induce DNA strand breaks *in vitro* and mutations in bacterial systems [11, 16, 17] free radicals generated by these Cr species at lower oxidation states may also be important in the mechanism of Cr(VI)-induced carcinogenicity [18]. This article will summarize studies from our laboratory on Cr(VI) reduction, free radical generation and cellular responses.

Reduction of Cr(VI)

Reduction of Cr(VI) by low molecular weight molecules

A variety of low molecular weight cellular constituents have been shown to reduce Cr(VI) *in vitro* at physiological pH. These substances include glutathione (GSH) [19, 20], cysteine [21], lipoic acid [22], and diol-containing molecules, such as

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NAD(P)H, ribose, fructose, and arabinose [23, 24]. Only those constituents which react with Cr(VI) at a significant rate are likely to contribute substantially to Cr(VI) reduction in cellular systems. Among these substances, ascorbate and GSH may be the most likely candidates as non-enzymatic Cr(VI) reductants, especially because of their ubiquitous occurrence in mammalian cells.

Reduction of Cr(VI) by GSH generates glutathionyl radical ($GS^{\bullet}$) [19, 20] and Cr(V) as well as Cr(IV) complexes [24, 25]. Both of these complexes can be isolated in solid forms [19, 24]. These stable Cr(V) and Cr(IV) solids can be used as model compounds to study the role of intracellular Cr(IV) and Cr(V) in the mechanism of Cr(VI)-induced carcinogenesis.

Another important Cr(VI) reductant is ascorbate [10]. Reduction of Cr(VI) by ascorbate is kinetically favored over GSH and intratracheal injection of Cr(VI) has been reported to deplete ascorbate but not GSH in rat lung [26]. In non-cellular systems, reduction of Cr(VI) by ascorbate has been reported to generate ascorbate-derived free radicals, Cr(V) and Cr(IV) [10]. The relative yield of these species was dependent on the relative concentrations of Cr(VI) and ascorbate. The Cr(V) and Cr(IV) generated by ascorbate reduction have been shown to react with H_2O_2 to produce hydroxyl radical ($^{\bullet}OH$), which caused DNA strand breaks [10].

Reduction of Cr(VI) by cellular reductant enzymes, organelles and intact cells

A variety of enzymatic and non-enzymatic factors function as Cr(VI) reductants. These factors include microsomes [27], mitochondria [27], and several flavoenzymes, such as glutathione reductase (GSSG-R), lipoyl dehydrogenase and ferredoxin-$NADP^+$ oxidoreductase [28–30]. Among these reductants, glutathione reductase is discussed here as an example. In the presence of NADPH, glutathione reductase reduces Cr(VI) to generate Cr(V), which was identified as a Cr(V)-NADPH complex. During the reduction process, molecular oxygen is reduced to $O_2^{\bullet-}$, which generates H_2O_2 via dismutation.

Our earlier study showed that incubation of *Escherichia coli*, mitochondria, or microsomes with $K_2Cr_2O_7$ generated a Cr(V)-NADPH complex [27]. Addition of NADPH enhanced the Cr(V)-NADPH formation, indicating that NADPH dependent reductases are likely to be involved in this Cr(V) formation.

Reduction of Cr(VI) in vivo

The most direct evidence for Cr(VI) reduction was demonstrated by the generation of Cr(V) in whole animals treated with Cr(VI) [31]. Using ESR with a low-frequency microwave bridge and a cylinder-shaped loop gap resonator, we were able to show that a Cr(V) intermediate can be generated by one-electron reduction of Cr(VI) in whole living animals [31]. The Cr(V) was found predominantly in the liver with a small amount in the blood. Liver homogenates from Cr(VI) treated animals generated essentially the same Cr(V) species as that obtained from the whole living mice. This Cr(V) species was identified to be a Cr(V)-NADPH complex with an oxygen bond to Cr(V) [32, 33]. Pretreatment of the animals with ascorbate or GSH decreased the Cr(V) formation, while pretreatment with NADPH enhanced it. Metal chelators, such as 1,10-phenanthroline and diethylenetriaminephentaacetic acid (DTPA), inhibited the formation of the Cr(V)-NADPH complex. These results suggest that NADPH/flavoenzymes and not GSH or ascorbate are the likely major one-electron Cr(VI) reductants *in vivo*.

Free radical generation

Thiyl radical generation

Using ESR spin trapping, Shi and Dalal [19] detected the formation of Cr(V) and glutathione-derived thiyl radicals ($GS^{\bullet}$) in the reaction of Cr(VI) with GSH. An increase in GSH concentration enhanced the $GS^{\bullet}$ radical generation. Reaction of Cr(VI) with cysteine or penicillamine also generates corresponding thiyl radicals [21]. The thiyl radicals generated by this reaction may cause direct cellular damage. These radicals may also react with other thiol molecules to generate $O_2^{\bullet-}$ radicals (Equations (1) and (2) [34, 35].

$$GS^{\bullet} + RSH \rightarrow RSSR^{\bullet-} + H^+ \quad (1)$$

$$RSSR^{\bullet-} + O_2 \rightarrow RSSR + O_2^{\bullet-} \quad (2)$$

The generation of $O_2^{\bullet-}$ radicals leads to the formation of H_2O_2. $O_2^{\bullet-}$ is able to cause additional oxygen radical generation, for example, by reducing Cr(VI) to Cr(V) and a subsequent reaction with H_2O_2 as discussed in the following sections.

Hydroxyl radical generation by Cr(V) reaction

Shi and Dalal [24] have reported that addition of H_2O_2 to a mixture of GSH and Cr(VI) decreased the ESR signal of Cr(V) and generated $^{\bullet}OH$ radicals. The $^{\bullet}OH$ generation was believed to be the result of reaction of Cr(V) with H_2O_2 via a Fenton-like reaction (Equation (3)).

$$Cr(V) + H_2O_2 \rightarrow Cr(VI) + {}^{\bullet}OH + OH^- \quad (3)$$

Cr(V)-NADPH generated by reaction of Cr(VI) with NADPH was used to verify the above reaction [23, 24, 29, 36]. A mixture of Cr(VI), NADPH and H_2O_2 generated both Cr(V) and $^{\bullet}OH$ radical. An increase in H_2O_2 concentration enhanced $^{\bullet}OH$ generation with a concomitant decrease in the Cr(V) formation. The reactivity of Cr(V) species depends on their structure [37]. For example, tetraperoxochromate $\{[Cr(O_2^{2-})_4]^{3-}\}$ has a tetrahederal structure with all covalent bonds fully occupied by $O_2^{\bullet-}$ moieties. The $[CR(O_2^{2-})_4]^{3-}/H_2O_2$ complex does not easily split H_2O_2 to generate $^{\bullet}OH$ radical. On the other hand, a Cr(V) complex, such as Cr(V)-NADPH, is octahedral, with one vacant site. H_2O_2 can attach to the vacant coordination site and form a long-lived complex to generate $^{\bullet}OH$ radicals.

Hydroxyl radical generation by Cr(IV) reaction

Cr(IV) is the other reactive chromium intermediate generated in the reduction of Cr(VI) by cellular reductants, such as ascorbate and GSH. Shi *et al*. [10] have demonstrated that Cr(IV) is able to generate $^{\bullet}OH$ radical from H_2O_2 via a Fenton-like reaction (Equation (4)).

$$Cr(IV) + H_2O_2 \rightarrow Cr(V) + {}^{\bullet}OH + OH^- \quad (4)$$

Reaction of Cr(VI) with ascorbate was used as a source of Cr(IV). The following experimental observations [10] support the validity of Equation (4): (i) A mixture of Cr(VI) and ascorbate generated both Cr(V) and Cr(IV) intermediates; and (ii) addition of H_2O_2 produced $^{\bullet}OH$ radicals with a significant enhancement of Cr(V) generation. In a system with both Cr(V) and Cr(IV) present, H_2O_2 reacted with both Cr(V) and Cr(IV) competitively. If the reaction rate of Cr(IV) with H_2O_2 were larger, the Cr(V) signal could increase upon addition of H_2O_2. Otherwise it would decrease. The observed enhancement of Cr(V) upon addition of H_2O_2 indicates that the reaction between Cr(IV) and H_2O_2 occurs and that the rate constant is larger than that of Cr(V) with HA. (iii) Synthesized Cr(IV) compounds, such as Cr(IV)-GSH and Cr(IV) ester of 2,4-dimethyl-2,4-pentanediol, were used to show that Cr(IV) was indeed able to generate $^{\bullet}OH$ radicals via a Fenton-like reaction [8, 25].

Once Cr(V) is generated, it reacts with H_2O_2 to generate more $^{\bullet}OH$ radicals. By adding Equations (3) and (4), the net reaction becomes that described in Equation (5).

$$Cr(IV) + 2H_2O_2 \rightarrow Cr(VI) + 2{}^{\bullet}OH + 2OH^- \quad (5)$$

Hydroxyl radical generation by Cr(III) reaction

The lack of toxicity and carcinogenicity of Cr(III) in intact cells can be explained by the poor uptake of this cation into cells. However, cells are permeable to Cr(VI). Once inside cells, Cr(VI) is eventually reduced to Cr(III). Our earlier studies have shown that Cr(III) is also able to generate $^{\bullet}OH$ radical from H_2O_2 [38]. The $^{\bullet}OH$ radical yield depends on pH, reaching the highest value at pH 10. This pH dependence indicates that high pH may promote oxidation of Cr(III) with H_2O_2. The latter is consistent with the observed decrease of Cr(III) concentration upon addition of H_2O_2 with a concomitant increase in $^{\bullet}OH$ radical generation.

Hydroxyl radical generation by a Cr(VI)- and Cr(III)-mediated Haber-Weiss reaction

Shi and Dalal [39] have studied the role of $O_2^{\bullet-}$ radical in Cr(VI)-generated $^{\bullet}OH$ radicals via a Cr(VI)-dependent Haber-Weiss cycle. Xanthine and xanthine oxidase were used as a source of $O_2^{\bullet-}$ radicals. The results show that $O_2^{\bullet-}$ was able to reduce Cr(VI) to Cr(V) (Equation (6)), which in turn reacted with H_2O_2 to generate $^{\bullet}OH$ and Cr(VI) (Equation (3)).

$$O_2^{\bullet-} + Cr(VI) \rightarrow Cr(V) + O_2 \quad (6)$$

By combining (3) and (6), we obtain (7):

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{Cr(VI)/Cr(V)} O_2 + {}^{\bullet}OH + OH^- \quad (7)$$

Similar to Cr(VI), we have shown that Cr(III) is also able to generate $^{\bullet}OH$ radicals via the Haber-Weiss cycle [40].

The metal chelators, deferoxamine, 1,10-phenanthroline and EDTA, decreased the $^{\bullet}OH$ generation, showing that proper coordination of Cr(III) is required for Cr(III)-mediated $^{\bullet}OH$ generation. The Haber-Weiss mechanism of $^{\bullet}OH$ generation could become particularly significant during phagocytosis when macrophages and their cellular constituents generate a large quantity of $O_2^{\bullet-}$ radicals during respiratory burst. It has been reported that a significant portion of oxygen consumed by phagocytes is converted to $O_2^{\bullet-}$ [41]. However, further conversion of $O_2^{\bullet-}$ to $^{\bullet}OH$ is too slow to be physiologically significant, unless a suitable metal ion (i.e. Fe(II)) is present as a Haber-Weiss catalyst [42]. The finding that Cr(VI) or Cr(III) can act as a Haber-Weiss catalyst may provide a basis for the known critical role of molecular oxygen in the genotoxic and carcinogenic reactions of Cr(VI)-containing particles.

ROS generation in intact cells

Using an ESR spin trapping technique, we have studied $^{\bullet}OH$ radical generation from intact (Jurkat) cells exposed to Cr(VI) [43]. Both $^{\bullet}OH$ and Cr(V) were produced from this system.

The Cr(V) generated was identified as a Cr(V)-NADPH complex. Addition of NADPH or glutathione reductase enhanced the Cr(V) generation, indicating that NADPH-dependent flavoenzymes were likely to be involved in the formation of the Cr(V)-NADPH complex. Addition of H_2O_2 enhanced $^{\bullet}OH$ radical generation and also increased the ESR signal intensity of Cr(V) peak. The observed enhancement of Cr(V) formation upon addition of H_2O_2 indicates that in this cellular system Cr(IV) also contributes to the Cr(VI)-induced $^{\bullet}OH$ generation.

Using confocal microscopy in combination with specific fluorescent dyes, our laboratories [43, 44] have also shown that Cr(VI)-exposed human lung epithelial cells (A549) generated H_2O_2 and $O_2^{\bullet-}$ which were inhibited by catalase and superoxide dismutase (SOD), respectively.

Role of free radical reactions in Cr(VI)-induced carcinogenesis

DNA damage

Using λ Hind III DNA digest, our laboratory assessed DNA damage induced by a mixture of Cr(VI) and ascorbate with and without H_2O_2 [10]. DNA strand breaks were detected by agarose gel electrophoresis. A significant amount of DNA strand breaks occurred when the DNA was incubated with Cr(VI) and ascorbate. The amount of DNA strand breaks depended on the relative concentrations of Cr(VI) and ascorbate. Addition of H_2O_2 drastically enhanced the DNA damage. Addition of Mn(II), which can remove Cr(IV) and inhibit the Cr(IV)-mediated Fenton-like reaction, inhibited DNA damage. The amount of DNA strand breaks correlated with the amount of free radicals generated.

$^{\bullet}OH$ radical can interact with guanine residues at several positions to generate a range of products, of which the most studied one is 8-hydroxy-deoxyguanosine (8-OHdG) [45]. The formation of this adduct is considered a marker to implicate ROS in the mechanism of toxicity and carcinogenicity of a variety of agents. Using high performance liquid chromatography (HPLC) with electrochemical detection, our previous studies found that $^{\bullet}OH$ radicals generated by Cr(V)- and Cr(IV) mediated reactions caused 2′-deoxyguanosine (dG) hydroxylation to form 8-OHdG [9, 10].

Using single cell gel electrophoresis, our laboratories have shown that Cr(VI) is able to cause DNA damage in the human prostate cell line, LNCap. Cr(VI)-induced DNA damage was stronger in Ras (+) cells, which overexpress Ras protein than a wild type [46]. Ras (+) cells are more potent in ROS generation under Cr(VI) stimulation than the wild type [46].

NF-κB activation

NF-κB is considered a primary oxidative stress response transcription factor that functions to enhance the transcription of a variety of genes [47–49]. Cr(VI) was able to induce NF-κB activation in Jurkat cells [50]. The reduction of Cr(VI) to low oxidation states is required for Cr(VI)-induced NF-κB activation. Hydroxyl radicals generated by Cr(V)- and Cr(IV)-mediated Fenton-like reactions play a prominent role in the mechanism of Cr(VI)-induced NF-κB activation. It may be noted that NF-κB binding sites serve as an enhancer element in the *c-myc* oncogene and this gene is associated with the formation of Burkitt's lymphoma [51]. Cr(VI) could induce expression of c-myc proto-oncogene via NF-κB activation. It is possible that NF-κB activation and a subsequent expression of proto-oncogenes, such as *c-myc*, may play a role in the induction of neoplastic transformation by Cr(VI).

AP-1 activation

Another important transcription factor whose activity is stimulated by Cr(VI) is AP-1. This is a dimeric, sequence-specific DNA binding protein composed of *jun* and *fos* products. Activation of AP-1 results in the over expression of *c-jun* and other proto-oncogenes. A number of mitogen activated protein kinases (MAPK) members participate in the activation of AP-1 hierarchically through divergent kinase cascades. MAPK, such as c-Jun-N-terminal kinase (JNK) and p38, are activated by a specific MAPK kinase (MAPKK) through phosphorylation of conserved threonine and tyrosine residues in JNK and p38. A MAPKK is activated by a specific MAPKK kinase (MAPKKK) through phosphorylation of conserved threonine and/or serine residues. Our laboratory has recently demonstrated that Cr(VI) is capable of inducing AP-1 activation [52]. The induction of AP-1 by Cr(VI) is associated with phosphorylation of MAP kinase p38 and JNK, but not extracellular-signal-regulated kinase (ERK). Aspirin, an antioxidant, inhibits the activation of AP-1 and NF-κB induced by Cr(VI). Inhibition of p38 and IκB kinase (IKK) attenuated Cr(VI)-induced AP-1 and NF-κB, respectively. These results suggest that ROS may serve as a common upstream signal initiating the activation of both AP-1 and NF-κB in response to Cr(VI) stimulation, whereas p38 and JNK act as a downstream executive kinase for the activation of AP-1 and NF-κB, respectively.

p53 Activation

The tumor suppressor protein p53 plays an important role in protecting cells from tumorgenic alternation. It has been re-

ported that more than 50% of human cancers contain mutations in the p53 gene. This transcription factor is considered as one of the oxidative stress response transcription factors and can be activated in response to a variety of stimuli, such as UV, γ radiation, and nucleotide deprivation. Our laboratory has shown that Cr(VI) is able to activate p53 in human lung epithelial cells (A549) by increasing the protein level and enhancing both DNA binding activity and transactivation ability of the protein [44, 53]. SOD, by enhancing the production of H_2O_2 from $O_2^{\cdot-}$, increased p53 activity. Catalase, a H_2O_2 scavenger, eliminated $^{\cdot}OH$ radical generation and inhibited p53 activation. Sodium formate and aspirin, $^{\cdot}OH$ radical scavengers, also suppressed p53 activation. Deferoxamine, a metal chelator, inhibited p53 activation by chelating Cr(V) to make it incapable of generating radicals from H_2O_2. NADPH, which accelerated the one-electron reduction of Cr(VI) to Cr(V) and increased $^{\cdot}OH$ radical generation, enhanced p53 activation. Thus $^{\cdot}OH$ radicals generated from Cr(VI) reduction in A549 cells are responsible for Cr(VI)-induced p53 activation.

Our laboratory has also shown that the activation of p53 is at the protein level instead of the transcriptional level [53]. The degradation of p53 was dramatically decreased upon stimulation by Cr(VI). In addition, Cr(VI) treatment decreased the interaction of p53 with mdm2 proto-oncoprotein, which blocks the transactivation ability of p53 and promotes the degradation of p53 protein. In response to Cr(VI) treatment, p53 protein becomes phosphorylated and acetylated at Ser15 and Lys383, respectively. The phosphorylation levels at either Ser20 or Ser392 did not show any significant alternation. It appears that Ser15 may play a key role in the dissociation of mdm2 in response to Cr(VI). Erk, a member of MAPK, acts as the upstream kinase for the phosphorylation of the p53 Ser15 site.

HIF-1 and VEGF induction

Vascular endothelial growth factor (VEGF) is an essential protein for tumor angiogenesis. Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric basic-helix-loop-helix transcription factor, composed of HIF-1α and HIF-1β/ARNT subunits. HIF-1α is unique to HIF-1 and is induced exponentially in response to a decrease in cellular O_2 concentration. HIF-1β is identical to the aryl hydrocarbon nuclear translocator (ARNT) that heterodimerizes with an aryl hydrocarbon receptor and is not regulated by cellular oxygen tension. HIF-1 regulates the expression of many genes including VEGF, erythropoietin (EPO), heme oxygenase 1, aldolase, enolase, and lactate dehydrogenase A. The levels of HIF-1 activity in cells are correlated with tumorigenicity and angiogenesis in nude mice. HIF-1 is induced by the expression of oncogenes, such as v-Src and Ras, and is overexpressed in many human cancers. HIF-1 activates the expression of VEGF gene at the transcriptional level. VEGF plays a key role in tumor progression and angiogenesis. Both inhibition of VEGF expression and function of its receptor dramatically decreases tumor growth, invasion, and metastasis in animal models. Tissue hypoxia is a major inducer for the expression of VEGF in tumors. Somatic mutations, such as oncogene Ras activation and tumor suppressor gene p53 inactivation, also increased VEGF expression. In a recent study [54], we have demonstrated that Cr(VI) induces HIF-1 activity through the specific expression of HIF-1α but not the HIF-1β subunit and increases the level of VEGF expression in DU145 human prostate carcinoma cells. To dissect the signaling pathways involved in Cr(VI)-induced HIF-1 expression, we found that p38 MAP kinase signaling was required for HIF-1α expression induced by Cr(VI). Neither PI 3K nor ERK activity was required for Cr(VI)-induced HIF-1 expression. Cr(VI) induced expression of HIF-1 and VEGF through the production of reactive oxygen species (ROS) in DU145 cells. The major species of ROS for the induction of HIF-1 and VEGF expression is H_2O_2. These results suggest that the expression of HIF-1 and VEGF induced by Cr(VI) may be an important signaling pathway in Cr(VI)-induced carcinogenesis.

Tyrosine phosphorylation

Tyrosine phosphorylation is an important step in the regulation of many key cellular functions. It is involved in control of cell proliferation, differentiation, cell-cycle regulation, cell signal transduction, metabolism, transcription, morphology, adhesion, ion channels, and cancer development. We have recently shown that Cr(VI) increased tyrosine phosphorylation in human epithelial A549 cells in a time-dependent manner [55]. N-acetyl-cysteine (NAC), a general antioxidant, inhibited Cr(VI)-induced tyrosine phosphorylation. Catalase (a scavenger of H_2O_2), sodium formate and aspirin (scavengers of $^{\cdot}OH$ radical) also inhibited the increased tyrosine phosphorylation induced by Cr(VI). H_2O_2 and $^{\cdot}OH$ radicals generated by cellular reduction of Cr(VI) are responsible for the increased tyrosine phosphorylation induced by Cr(VI).

Apoptosis

Apoptosis is a process in which cell death is initiated and completed in an orderly manner through activation and/or synthesis of gene products necessary for cell destruction. It is a response to physiological and pathological stresses that disrupt the balance between the rates of cell division and elimination. In diseases such as cancer, there is an imbalance between the rate of cell division and death, influencing the anomalous accumulation of neoplastic cells. We have shown

that Cr(VI) is able to cause apoptosis [43]. In the apoptotic signaling pathway, ROS generated from both Cr(VI) reduction and p53 activation play an important role. The Cr(VI)-derived ROS initiate apoptosis before activation of p53 protein. Although p53 is not required for initiation of Cr(V)-induced apoptosis, it may enhance apoptosis by transcriptional activation of a redox-related gene. Cr(VI) induces apoptosis through both p53-dependent and p53-independent pathways. ROS generated by Cr(VI) may play a dual role in the mechanism of Cr(VI)-induced carcinogenesis: genetic damage and apoptosis. The Cr(VI)-induced carcinogenesis may depend on the balance of these two opposite processes.

Cell cycle arrest

Under normal circumstances the cell cycle proceeds without interruptions. However, when damage occurs particularly to DNA, most normal cells have the capacity to arrest proliferation in the G_1/S or G_2/M phase and then resume proliferation after the damage is repaired. The cell cycle controls the onset of DNA replication and mitosis in order to ensure the integrity of the genome. Lack of fidelity in DNA replication and maintenance can result in mutations, leading to cell death or, in multicellular organisms, cancer. Using flow cytometric analysis of DNA content, our laboratory [56] has shown that (a) Cr(VI) is able to induce cell cycle arrest at the G_2/M phase in human lung epithelial A549 cells; (b) while at relatively low concentrations Cr(VI) causes cell cycle arrest, at relatively high concentrations Cr(VI) induces apoptosis; and (c) ROS generated by Cr(VI)-stimulated cells are involved in Cr(VI)-induced cell cycle arrest and among the ROS H_2O_2 plays a key role.

Gene expression profile

Recent advances in high-through-put-screening technology has made it possible to analyze expression of thousands of genes at a time. Oligonucleotide-based micro-array represents a state-of-the-art approach in large-scale screening technology. Our recent study used genechip technology to analyze differential gene expression in response to Cr(VI)-induced cell stress [57]. The results show that many oxidative stress-related genes are induced in response to Cr(VI) treatment. These genes include Cu/Zn SOD, glutathione peroxidase and metallothionein IIA. Several calcium-related genes were induced by Cr(VI), for example, calcineurin A2 and caldesmon. Since the calcium signal is coupled with ROS generation, induction of calcium-related genes becomes indirect evidence that Cr(VI) induces oxidative stress in the cells. Cr(VI) also induces several important genes in intracellular kinase cascades. The molecules corresponding to these genes include G-protein, Src kinase and MAPK. G-protein and Src-kinase are key proteins in mediating receptor signals in the cells. In mammals, both G-proteins and Src-kinase are involved in the regulation of cell proliferation and differentiation. Cr(VI) induces various oncogenes, such as Jun-B and raf oncogenes, and genes involved in cell cycle regulation, such as cyclin-dependent kinases.

Conclusions

Cr(VI) can be reduced by various cellular reductants to its lower oxidation states, such as Cr(V) and Cr(VI). During the reduction process, molecular oxygen is reduced to $O_2^{\bullet-}$, which reduces to H_2O_2 upon dismutation. H_2O_2 reacts with Cr(V) or Cr(IV) to generate $^{\bullet}OH$ radicals. Thus Cr(VI) is able to generate a whole spectrum of ROS. Biological systems are normally protected against oxidative injury caused by free radical reactions by enzymatic and non-enzymatic antioxidants. When the balance between pro-oxidants and antioxidants shifts in favor of pro-oxidants, chromium-induced oxidative injury occurs. Through ROS-mediated reactions, Cr(VI) causes DNA damage, activation of nuclear transcription factors (NF-κB, AP-1, and p53), induction of apoptosis, over-expression of oncogenes, up-regulation of antioxidants, activation of certain enzymes involved in MAPK signal pathways, stimulation of enzymes involved in cell cycle control and checkpoint mechanisms, and activation of enzymes responsible for Cr(VI) reduction, such as NADPH dependent dihydrolipoamide dehydrogenase.

References

1. Cohen MD, Kargacin B, Klein CB, Costa M: Mechanism of chromium carcinogenicity and toxicity. Crit Rev Toxicol 23: 255–281, 1993
2. Costa M: Toxicity and carcinogenicity of Cr(VI) in animal models and humans. Crit Rev Toxicol 27: 431–442, 1997
3. IARC: Chromium and chromium compounds: Chromium metal (group 3) trivalent compounds (group) hexavalent chromium compounds (group 1). IARC Monogr Eval Carcinogen Risk Chem Hum 7(suppl): 165–168, 1987
4. IARC: Chromium, nickel and welding. IARC Monogr Eval Carcinogen Risk Chem Hum 49: 55–62, 1990
5. De Flora S, Bagnasco M, Serra D, Zanacchi P: Genotoxicity of chromium compounds: A review. Mutat Res 238: 99–172,1990
6. De Flora S, Wetterhahn KE: Mechanisms of chromium metabolism and genotoxicity. Life Chem Rep 7: 169–244, 1989
7. Sugiyama M: Role of physiological antioxidants in chromium(VI)-induced cellular injury. Free Radic Biol Med 12: 397–407, 1992
8. Luo H, Lu YD, Shi X, Mao Y, Dalal NS: Chromium(IV)/H2O2 Fenton-like reaction causes DNA damage: Implication to chromate's genotoxicity. Ann Clin Lab Sci 26: 185–191, 1996
9. Shi X, Sun X, Gannett PM, Dalal NS: Deferoxamine inhibition of Cr(V)-mediated radical generation and deoxyguanine hydroxylation: ESR and HPLC evidence. Arch Biochem Biophys 293: 281–286, 1992

10. Shi X, Mao Y, Knapton AD, Ding M, Rojanasakul Y, Gannett PM, Dalal NS, Liu K: Reaction of Cr(VI) with ascorbate and hydrogen peroxide generates hydroxyl radicals and causes DNA damage: Role of Cr(IV)-mediated Fenton-like reaction. Carcinogenesis 15: 2475–2478, 1994
11. Standeven AM, Wetterhahn KE: Possible role of glutathione in chromium(VI) metabolism and toxicity in rats. Pharmacol Toxicol 68: 469–476, 1991
12. Connett PH, Wetterhahn KE: Metabolism of the carcinogenic chromate by cellular constituents. Struct Bond 54: 93–124, 1983
13. Kortenkamp A, Beyersmann D, O'Brien P: Uptake of chromium(III) complexes by erythrocytes. Toxicol Environ Chem 14: 23–29, 1987
14. Tsapakos MJ, Wetterhahn KE: Interaction of chromium with nucleic acids Chem Biol Interact 46: 265–277, 1983
15. Jennett KW: Microsomal reduction of the carcinogen chromate produces chromium(V). J Am Chem Soc 104: 874–875, 1982
16. Kortenkamp A, Ozolins Z, Beyersmann D, O'Brien P: Generation of PM2 DNA breaks in the course of reduction of chromium(VI) by glutathione. Mutat Res 216: 19–26, 1989
17. Rodney PF, Robert JJ, Lay PA, Dixon NE, Raker RSU, Bonin AM: Chromium(V)-induced cleavage of DNA: Are chromium(V) complexes the active carcinogens in chromium(VI)-induced cancer? Chem Res Toxicol 2: 227–229, 1989
18. Shi X, Chiu A, Chen CT, Halliwell B, Castranova V, Vallyathan VJ: Reduction of chromium(VI) and its relationship to carcinogenesis. Toxicol Environ Health 2: 101–118, 1998
19. Shi X, Dalal NS: On the mechanism of the chromate reduction by glutathione: ESR evidence for the glutathionyl radical and an isolable Cr(V) intermediate. Biochem Biophys Res Commun 156:137–142, 1988
20. Dalal NS, Shi X: ESR evidence for the glutathionyl radical in the reduction of chromium(VI) by glutathione. In: Medical, Biomedical and Chemical Aspects of Free Radicals. Elsevier Science Publishers, Amsterdam, 1989, pp 547–550
21. Shi X, Dong Z, Dalal NS, Gannett P: Chromate-mediated free radical generation from cysteine, penicillamine, hydrogen peroxide, and lipid hydroperoxides. Biophys Acta 1226: 65–72, 1994
22. Chen F, Ye J, Zhang X, Rojanasakul Y, Shi X: One-electron reduction of chromium(VI) by alpha-lipoic acid and related hydroxyl radical generation, dG hydroxylation and nuclear transcription factor-κB activation. Arch Biochem Biophys 338: 165–172, 1997
23. Shi X, Dalal NS: ESR spin trapping detection of hydroxyl radicals in the reaction of Cr(V) complexes with hydrogen peroxide. Free Radic Res Commun 10: 17–26, 1990
24. Shi X, Dalal NS: On the hydroxyl radical formation in the reaction between hydrogen peroxide and biologically generated chromium(V) species. Arch Biochem Biophys 277: 342–350, 1990
25. Liu KJ, Shi X, Dalal NS: Synthesis of Cr(VI)-GSH, its identification and its free hydroxyl generation: A model compound for Cr(VI) carcinogenicity. Biochem Biophys Res Commun 235: 54–58, 1997
26. Suzuki Y, Fukuda K: Reduction of hexavalent chromium by ascorbic acid and glutathione with special reference to the rat lung. Arch Toxicol 64: 169–176, 1994
27. Shi X, Dalal NS, Vallyathan V: One electron reduction of carcinogen chromate by microsomes, mitochondria and *E. coli*: Identification of Cr(V) and ·OH radical. Arch Biochem Biophys 290: 381–386, 1991
28. Shi X, Dalal NS: Chromium(V) and hydroxyl radical formation during the glutathione reductase-catalyzed reduction of chromium(VI). Biochem Biophys Res Commun 163: 627–634, 1989
29. Shi X, Dalal NS: NADPH dependent flavoenzymes catalyze one electron reduction of metal ions and molecular oxygen and generate hydroxyl radical. FEBS Lett 276: 189–191, 1990
30. Shi X, Dalal NS: Free radical generation by mineral ions adsorbed on mineral dust: A mechanism for chronic lung injury. Respirable dust in the mineral industries. Society for Mining, Metallurgy and Exploration, Inc., Littleton, Colorado, 38: 307–311, 1991
31. Liu K, Jiang J, Swartz HM, Shi X: Low frequency EPR detection of chromium(V) formation by chromium(VI) reduction in whole live mice. Arch Biochem Biophys 313: 248–255,1995
32. Liu KJ, Shi X, Jiang JJ, Goda F, Dalal NS, Swartz HM: Chromate-induced chromium(V) formation in live mice and its control by cellular antioxidants: An L-band EPR study. Arch Biochem Biophys 323: 33–39, 1995
33. Liu KJ, Shi X, Jian JJ, Doda F, Dalal NS, Swartz HM: Low frequency electron paramagnetic resonance investigation on metabolism of chromium(VI) by whole mice. Annu Clin Lab Sci 26:176–184, 1996
34. Barton JP, Packer JE: Radiolysis of oxygenated cysteine solutions at neutral pH. The role of RSSR and O_2^-. Int J Rad Phys Chem 23: 159–166, 1970
35. Quintiliani M, Badiello R, Tamba M, Esfendi A, Gorin G: Radiolysis of glutathione in oxygen-containing solutions of pH 7. Int J Rad Phys Chem 32: 159–166, 1997
36. Shi X, Dalal NS: Evidence for a fenton-type mechanism for the generation of ·OH radicals in the reduction of Cr(VI) in cellular media. Arch Biochem Biophys 281: 90–95, 1990
37. Shi X, Dalal NS: On the mechanism of generation of hydroxyl radical by chromate in biologically relevant systems: Role of Cr(V) complexes vs. tetraperoxochromate(V). Environ Health Perspect 102: 231–236, 1994
38. Shi X, Dalal NS, Kasprzak KS: Generation of free radicals from hydrogen peroxide and lipid hydroperoxides in the presence of Cr(III). Arch Biochem Biophys 302: 294–299, 1993
39. Shi X, Dalal NS: The role of superoxide radical in chromium(VI) generated hydroxyl radical: The Cr(VI) Haber-Weiss cycle. Arch Biochem Biophys 292: 323–327, 1992
40. Shi X, Leonard S, Zang L, Gannett P, Rojanasakul Y, Castranova V, Vallyathan V: Cr(III)-mediated hydroxyl radical generation via Haber-Weiss cycle. J Inorg Biochem 69: 263–268, 1998
41. Freeman BA, Crapo JD: Biology and disease: Free radicals and tissue injury. Lab Invest 47: 412–426, 1982
42. Weinstein J, Bielski BHJ: Kinetics of the interaction of H_2O_2 and O_2^- radicals with hydrogen peroxide. J Am Chem Soc 101: 58–62, 1979
43. Ye J, Wang S, Leonard SS, Sun Y, Butterworth L, Antonini J, Ding M, Rojanasakul R, Vallyathan V, Castranova V, Shi X: Role of reactive oxygen species and p53 in chromium(VI)-induced apoptosis. J Biol Chem 274: 34974–34980, 1999
44. Wang S, Leonard SS, Ye J, Ding M, Shi X: The role of hydroxyl radical as a messenger in Cr(VI)-induced p53 activation. Am J Physiol C868–C875, 2000
45. Dizadaroglu M: Chemical determination of free radical-induced damage to DNA. Free Radic Biol Med 10: 225–242, 1991
46. Liu K, Husler J, Ye J, Leonard SS, Cutler D, Chen F, Wang S, Zhang Z, Ding M, Wang L, Shi X: On the mechanism of Cr(VI)-induced carcinogenesis: Dose dependence of cellular responses. Mol Cell Biochem 222: 221–229, 2001
47. Chen F, Castranova, V, Shi X, Demers LM: New insights into the role of NF-κB, a ubiquitous transcription factor in initiation of diseases. Clin Chem 45: 7–17, 1999
48. Baldwin AS: The NF-κB and IκB proteins: New discoveries and insights. Annu Rev Immunol 14: 649–681, 1996
49. Baeuerle PA, Henkel T: Function and activation of NF-κB in the immune system. Annu Rev Immunol 12: 141–179, 1994
50. Ye J, Zhang X, Young HA, Mao Y, Shi X: Chromium(VI)-induced nuclear factor-κB activation in intact cells via free radical reactions. Carcinogenesis 16: 2401–2405, 1995
51. Ji L, Arcinas M, Boxer LM: NF-κB sites function as positive regulators of expression of the translocated c-myc allelle in Burkitt's lymphoma. Mol Cell Biol 14: 141–179, 1994

52. Chen F, Ding F, Lu Y, Leonard SS, Vallyathan V, Castranova V, Shi X: Participation of MAP kinase p38 and IκB kinase in chromium(VI)-induced NF-κB and AP-1 activation. J Environ Pathol Toxicol Oncol 19: 231–238, 2000
53. Wang S, Shi X: Mechanism of Cr(VI)-induced p53 activation: The role of phosphorylation, mdm2 and ERK. Carcinogenesis 22: 757–762, 2001
54. Gao N, Jiang B, Leonard SS, Corum L, Zhang Z, Roberts JR, Antonini J, Zheng JZ, Flynn DC, Shi X: Induction of hypoxia-inducible factor 1 and vascular endothelial growth factor by Cr(VI) through reactive oxygen species in DU145 human prostate carcinoma cells (manuscript in preparation).
55. Qian Y, Jiang B, Leonard SS, Wang S, Zhang Z, Ye J, Chen F, Wang L, Flynn DC, Shi X: Cr(VI) increases tyrosine phosphorylation through reactive oxygen species-mediated reactions. Mol Cell Biochem 222: 199–204, 2001
56. Zhang Z, Leonard SS, Wang S, Vallyathan V, Castranova V, Shi X: Cr(VI) induces cell growth arrest through hydrogen peroxide-mediated reactions. Mol Cell Biochem 222: 77–83, 2001
57. Ye J, Shi X: Gene expression profile in response to chromium-induced cell stress in A549 cells. Mol Cell Biochem 222: 189–197, 2001