Genetic and Epigenetic Mechanisms in Metal Carcinogenesis and Cocarcinogenesis: Nickel, Arsenic, and Chromium

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Chronic exposure to nickel(II), chromium(VI), or inorganic arsenic (iAs) has long been known to increase cancer incidence among affected individuals. Recent epidemiological studies have found that carcinogenic risks associated with chromate and iAs exposures were substantially higher than previously thought, which led to major revisions of the federal standards regulating ambient and drinking water levels. Genotoxic effects of Cr(VI) and iAs are strongly influenced by their intracellular metabolism, which creates several reactive intermediates and byproducts. Toxic metals are capable of potent and surprisingly selective activation of stress-signaling pathways, which are known to contribute to the development of human cancers. Depending on the metal, ascorbate (vitamin C) has been found to act either as a strong enhancer or suppressor of toxic responses in human cells. In addition to genetic damage via both oxidative and nonoxidative (DNA adducts) mechanisms, metals can also cause significant changes in DNA methylation and histone modifications, leading to epigenetic silencing or reactivation of gene expression. In vitro genotoxicity experiments and recent animal carcinogenicity studies provided strong support for the idea that metals can act as cocarcinogens in combination with nonmetal carcinogens. Cocarcinogenic and comutagenic effects of metals are likely to stem from their ability to interfere with DNA repair processes. Overall, metal carcinogenesis appears to require the formation of specific metal complexes, chromosomal damage, and activation of signal transduction pathways promoting survival and expansion of genetically/epigenetically altered cells.

Contents

1. Introduction

The ability of some metal compounds to cause cancers in exposed workers has been known for a long time, with early documented cases dating back to the 19th century. Massive growth of manufacturing and other economic activities in the major industrialized countries has been accompanied by parallel increases in the large-scale consumption of nonferrous metals, some of which are now recognized human carcinogens. High-volume utilization and poor practices in the disposal of metal-containing waste products created numerous sources of heavy environmental contamination, including some of the largest toxic sites known as Superfund sites in the U.S. Toxic metals represent the ultimate form of persistent environmental pollutants because they are chemically and biologically indestructible. Despite well-recognized carcinogenic potentials of such toxic metals as chromium, nickel, and metalloid arsenic, the molecular mechanisms underlying their cell-transforming ability remain poorly understood. Carcinogenic metals are typically weak mutagens, and with the exception of chromium, they do not form DNA adducts, which represent a key initiating event in the cancer-inducing activity of organic carcinogens. A long-held view that elevated production of reactive oxygen species is the main pathway in metal carcinogenicity is clearly at odds with data on weak or no mutagenicity of most metals. This perspective will summarize the most recent development in the field of metal carcinogenicity and cocarcinogenicity with special emphasis on the roles of activated signaling pathways, epigenetic changes, and DNA repair processes.

2. Nickel

2.1. Human Exposure and Carcinogenicity. Nickel(II) is a toxic and carcinogenic metal (*1*). It is used in modern industry with other metals to form alloys to produce coins, jewelry, and stainless steel as well as for nickel plating and manufacturing

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Ni-Cd batteries. Among new applications, it is important to note its role as a catalyst for the production of carbon nanoparticles. This new technology increases consumption and contamination with nickel compounds. Workers are exposed at different stages of processing of nickel-containing products. The most important route of human exposure to nickel is inhalation. This exposure has long been known to cause acute respiratory symptoms, ranging from mild irritation and inflammation of respiratory system to bronchitis, pulmonary fibrosis, asthma, and pulmonary edema (*2*). Additionally, nickel exposure also may cause cardiovascular and kidney diseases, as well as allergic dermatitis. However, nickel carcinogenic activity represents the most serious concerns. Nickel(II) exerts its carcinogenic activity most likely through nongenotoxic mechanisms. The toxicity and carcinogenicity of Ni(II) depends on its intracellular dose that, in turn, is a function of physicochemical properties of particular nickel compounds, their ability to enter the cell and/or to dissolve within the cell. Because of a fast clearance from the exposed tissues, which limits cellular uptake, water-soluble Ni(II) compounds possess lower toxic and carcinogenic potential as compared to semisoluble compounds such as nickel subsulfide (*1*). Possible mechanisms of nickel carcinogenesis have been discussed in a number of comprehensive reviews (*1, 3–6*).

Epidemiological studies have clearly implicated Ni(II) compounds as human carcinogens on the basis of an increased mortality from respiratory tract malignancies in refinery workers chronically exposed to nickel-containing dusts and fumes (*7, 8*). Other health effects of inhalation exposure to soluble and insoluble nickel compounds are reported in a number of recent publications (*2, 4, 8–12*). Acute lung injury following nickel exposure was demonstrated in mice and rats (*13–16*). In various animal models, chronic exposure to nickel compounds induces tumors at virtually any site of administration (*1, 3*). Nickel compounds efficiently transform rodent and human cells *in vitro* (*17–20*). On the basis of these observations, the International Agency for Research on Cancer (IARC) evaluated the carcinogenicity of nickel in 1990 (*21*). All Ni(II) compounds were recognized as human carcinogens (Group 1), and metallic nickel is classified as possibly carcinogenic to humans (Group 2B) (*21*). The purpose of this perspective is to re-evaluate existing hypotheses on the basis of recently obtained data.

2.2. Genetic and Epigenetic Changes. Although various potentially mutagenic DNA lesions have been shown to occur following nickel exposure, the actual mutagenic activity of nickel compounds observed in most of the mutational systems examined thus far from *Salmonella* to mammalian cells *in vitro* has been low (*22–25*). Thus, it may be suggested that nickel-induced mutagenic activity is not the primary cause in nickel-induced carcinogenesis. Indeed, the experiments with the SHE system have provided confirmatory evidence that cell immortalization can occur as an indirect consequence of carcinogen exposure following an induced high frequency change in the treated population, rather than through direct targeted mutagenesis (*26*). Additionally, no increase of ouabain-resistant or 6-thioguanine-resistant colonies has been found in human diploid fibroblasts even at concentrations of Ni_3S_2 that increased the frequency of anchorage-independence by 200-fold (*17*). These data implicate epigenetic changes as primary events in nickel carcinogenesis, which may include changes in the histone acetylation, methylation, or ubiquitylation levels, structural changes, and/or alterations in DNA methylation as well as the activation or suppression of a number of transcription factors (*27–33*).

Changes in DNA methylation leading to the inactivation of gene expression following the exposure to nickel compounds were initially found using the transgenic *E. coli gpt* gene in Chinese hamster G12 cells as a model (*29*). Although the mechanisms by which nickel induces DNA hypermethylation in cultured cells are presently unknown, a possible model includes the ability of nickel to substitute for magnesium, increase chromatin condensation and trigger *de novo* DNA methylation (*29*). Changes in DNA methylation can also be observed *in vivo* in nickel-induced tumors. The injection of nickel sulfide into wild type C57BL/6 mice as well as a mouse heterozygous for the tumor suppressor gene, p53, produced malignant histiocytomas in all mice (*34*). The hypermethylation of the promoter of the tumor suppressor gene p16 was observed in all tumors. Fhit is another tumor suppressor gene silenced by nickel exposure both *in vitro* and *in vivo* (*35*). Fhit is a tumor suppressor gene whose expression is frequently reduced or lost in tumors and premalignant lesions. A decrease of up to >90% in FHIT protein levels was observed in 22 local sarcomas (mostly fibrosarcomas) induced by i.m. injection of nickel subsulfide in C57BL/6 and MT+ (C57BL/6 overexpressing metallothionein) mice, as compared with normal muscles. The lack of FHIT protein coincided with the absence of the Fhit-mRNA transcript in these tumors.

In addition to gene silencing by DNA methylation, the suppressive effects of nickel on histone H4 acetylation *in vitro* in both yeast and mammalian cells have been reported (*30, 33*). Acetylation of lysine 12 and 16 in yeast was more strongly affected than lysine 5 and 8, and it was proposed that nickel binding to histidine 18 in histone H4 may be responsible for this effect (*36*). The loss of histone acetylation and DNA methylation worked together in *gpt* gene silencing in G12 transgenic cell line by nickel (*32, 37*). In human lung cells exposed to soluble nickel compounds, three major changes in histone modifications were observed: (i) loss of acetylation of H2A, H2B, H3, and H4; (ii) increases of H3K9 dimethylation; and (iii) substantial increases in the ubiquitylation of H2A and H2B (*28, 30–33*). The acetylation of the core histone N-terminal tail domains is recognized as a highly conserved mechanism for regulating chromatin functional states. Biochemical data supports a correlation between histone acetylation and gene activation, suggesting that histone acetylation acts to enhance the access of transcription-associated proteins to DNA. Conversely, histone methylation results in more compacted chromatin and gene silencing. If gene silencing mediated by histone modification plays a role in nickel-induced cell transformation, then the reactivation of these genes may reverse this effect. Indeed, recent experiments showed that the exposure of nickel-transformed cells to the histone deacetylase inhibitor trichostatin A (TSA) resulted in the appearance of a significant number of revertants measured by their inability to grow in soft agar (*38*). Moreover, pretreatment of cells with TSA inhibited the ability of nickel to transform mouse PW or human HOS cells to anchorage-independent growth. Low levels of histone acetylation in nickel-exposed cells may result from low levels of acetyl CoA, which is a universal donor of acetyl group. This may occur because the conversion of pyruvate into acetyl-CoA is blocked by the enzyme pyruvate dehydrogenase kinase (Figure 1). Taken together, these data suggest that epigenetic changes probably are more important for nickel-induced toxic and carcinogenic effects than mutational changes.

2.3. Activation of Hypoxic Signaling. Nickel is not an essential metal ion in mammalian cells. Therefore, no specific proteins involved in uptake, intracellular distribution, or storage

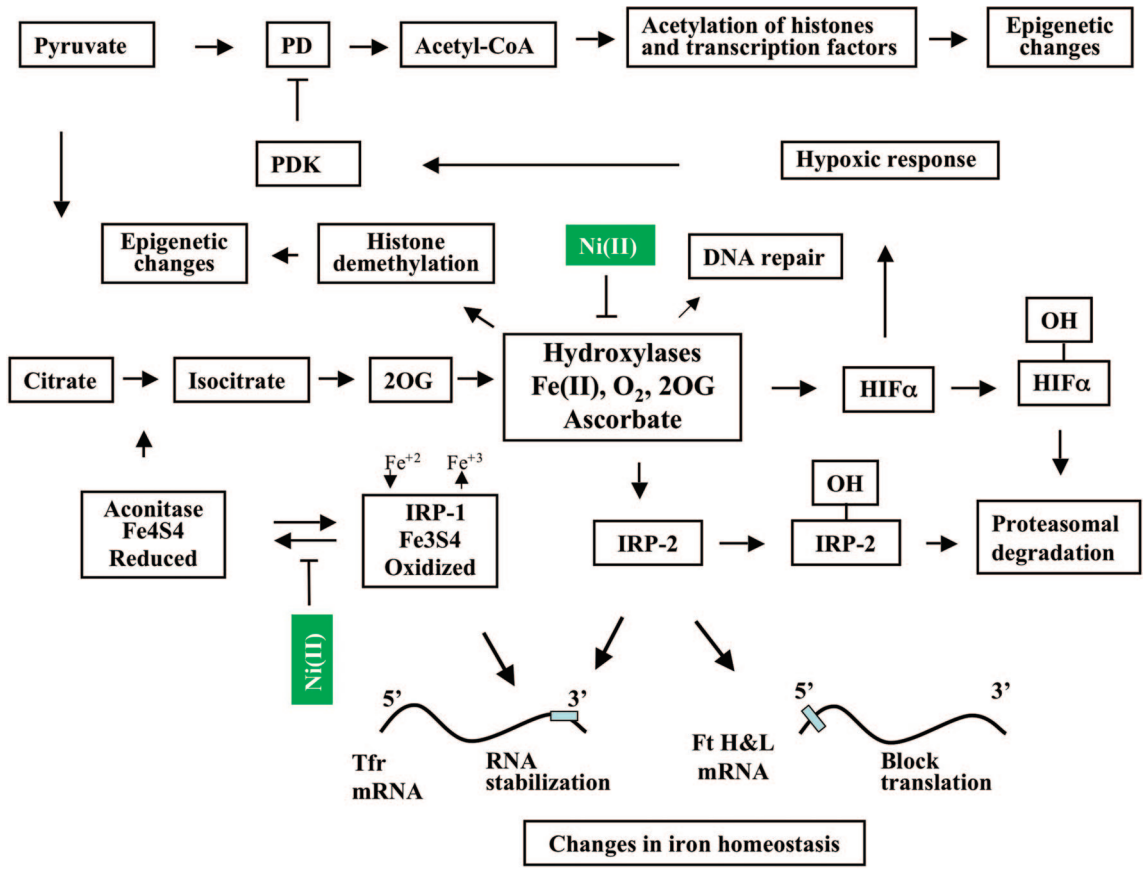

Figure 1. Schematic representation of major cellular interactions of Ni(II) derived from water-insoluble or water-soluble nickel compounds. Primary intracellular effect of Ni(II) is iron oxidation in iron-containing hydroxylases and iron-sulfur clusters. This occurs in part due to the depletion of intracellular ascorbate, which is a major iron reductant in cells. Iron oxidation in iron-containing hydroxylases results in the inhibition of their activity. Inhibition of HIF-prolyl hydroxylases 1–3 and HIF-asparaginyl hydroxyalse leads to HIFαs accumulation and activation of hypoxia-inducible genes (hypoxic response). Pyruvate dehydrogenase kinase (PDK) is one of the hypoxia-inducible genes. It inhibits pyruvate dehydrogenase (PD), which is critical for the conversion of pyruvate to acetyl-CoA and maintaining of acetyl-CoA levels. Since acetyl-CoA is a donor of the acetyl group, the decrease in the levels of acetyl-CoA will affect acetylation of histones and other transcription factors. Inhibition of other hydroxylases should lead to the loss of IRP-2 hydroxylation and IRP-2 stabilization. This along with oxidation of iron in IRP-1 result in transferrin receptor (Tfr) RNA stabilization and blocks ferritin heavy and light chain (Ft H&L) translation. Since transferrin receptor and ferritin are two major proteins in iron metabolism, this will affect intracellular iron homeostasis. Additionally, oxidation of iron in IRP-1 results in inactivation of aconitase. This in turn should inhibit the tricarboxylic acid cycle and affect levels of 2 oxoglutarate (2OG), a cofactor of hydroxylases. Recently discovered iron-containing 2OG-dependent hydroxylases (enzymes of the JMJD2 family), which can remove methyl groups from H3-K9me3 and H3-K36me3, also could be inhibited by Ni(II). This will impair the accessibility of DNA for transcription factors and, as the result, affect gene expression.

of nickel ions are known that may be commonly up-regulated by nickel exposure. Thus, no characteristic to nickel exposure pattern of gene expression was expected. Remarkably, nickel exposure produces a rather specific pattern of gene expression, which is similar to the response to hypoxia (*39–41*). The mechanism involves the inhibition of the HIF prolyl- and asparaginyl-hydroxylases, the stabilization of the HIFα proteins, and stimulation of HIF-1-dependent transcription (*39*). The HIF-1 transcription factor is a HIF-1α/HIF-1β (ARNT) dimer, which is formed in response to low oxygen tension in the cells. The α subunit is the regulatory component of the HIF-1 complex and is unique to the hypoxic response. Under normoxic conditions, this protein is virtually undetectable in most cells but can accumulate following exposure to proteasomal inhibitors, such as lactacystin or MG-132 (*42*). Accumulation of the HIFα subunit in the presence of hypoxia or nickel implies that proteasomal degradation of the protein is impaired by these exposures.

Hydroxylation of proline residues 402 and 564 in the presence of oxygen in the oxygen-dependent degradation domain (ODD) of HIF-1α leads to its interaction with the von Hippel Lindau (pVHL) tumor suppressor protein, a part of the ubiquitin–ligase complex (*43–45*). This results in ubiquitylation and rapid proteosomal degradation of HIF-1α. The structural basis for specific interaction of HIFα and VHL is provided by the introduction of the hydroxyl group at the proline C4-position. The oxygen of this hydroxyl group facilitates hydrogen bonding with VHL's Ser-111 and His-115 residues. This is sufficient to enable discrimination between nonhydroxylated and hydroxylated HIF-1α protein (*44–48*). In addition to proline hydroxylation, asparagine 803 hydroxylation, which allows for complex formation between HIF-1α and the CBP and p300 transcriptional coactivators, regulates transactivation of HIF-dependent genes (*49, 50*).

Nickel was found to be a strong inducer of HIF-1α protein and an activator of HIF-dependent transcription (*39, 51, 52*). Since Ni(II) is similar to Fe(II), it was suggested that Ni(II) could change iron metabolism and either replace Fe(II) in the hydroxylases or interfere with Fe(II) uptake (*53, 54*). These data were mainly supported by the finding that ferritin disappeared and IRP1 was activated in nickel-exposed cells (*55–57*). However, the oxidation of iron in iron–sulfur clusters could produce the same result (Figure 1). Modest changes in intracellular iron levels in nickel-exposed cells support this notion (*55*). Alternatively, it was suggested that reactive oxygen species (ROS) may play an important role in HIF-1α stabilization and

metals, which are known to generate free radicals in cells, and may stabilize HIF-1α through this mechanism (*58*). However, some data indicate that Ni(II) and perhaps other metals do not require free ROS generation for HIF-1α stabilization (*59, 60*). Recently, we showed that the depletion of intracellular ascorbate by Ni(II) may lead to the inhibition of prolyl hydroxylases (*39, 61*). These enzymes are members of the Fe(II)-, 2-oxoglutarate (2OG)-, and ascorbate-dependent family of dioxygenases. Ascorbate plays an important role in the reduction of enzyme-bound iron, which is vital for maintaining hydroxylase activity. The active role of ascorbate in the hydroxylase reactions may explain the controversy about the role of ROS in HIF-1 activation and reconcile previously obtained data. Indeed, ROS may deplete through oxidation a variety of reducing molecules. However, since only ascorbate is capable of maintaining iron in the reduced state, its presence is critical for hydroxylase activity. Ni(II) can deplete intracellular ascorbate via oxidation and/or by inhibiting ascorbate uptake by cells (*52, 61–63*). This results in the inactivation of the hydroxylases and produces a phenotype observed in hypoxic cells or in cells with mutated VHL (*61, 63*). Thus, exposure of cells to Ni(II) most likely results in the oxidation of intracellular iron followed by the induction of HIF-1 and activation expression of hypoxia-inducible genes (*39*).

The activation of hypoxic signaling pathway and switch of cellular metabolism to a state that mimics permanent hypoxia may be a part of nickel-induced carcinogenesis (*39, 64, 65*). Hypoxia is common in tumors because the tumor body is growing faster than the blood vessels growing into it. It may promote tumor progression via activation of genes coding for proteins, enabling cells to overcome nutritive deprivation and to escape from the hostile metabolic microenvironment (*66*). Stimulation of angiogenesis through up-regulation of VEGF and other growth factors involved in building new blood vessels is also an important part of the survival program. Additionally, cellular responses to hypoxic stress include inhibition of cell proliferation and, when cell damage is irreversible, apoptosis. Therefore, imitation of the state of hypoxia by nickel may provide the conditions for the selection of cells that have altered energy metabolism, changed growth control and/or have become resistant to apoptosis. The activation of HIF-1 transcription factor and modification of histones may represent a molecular basis for cellular adaptation in growing tumors. The selection theory seems to explain the low mutagenic but high transforming activity of nickel compounds (*26*). However, one may suggest that for successful cell transformation an additional mutagenic (DNA damage) event is required.

2.4. Ni(II) as a Cocarcinogen. Nickel compounds were shown to act synergistically with many mutagenic carcinogens in enhancing cell transformation both *in vitro* and *in vivo* (*67–69*). A single i.p. injection of nickel(II) acetate followed by subsequent dosing with sodium barbital in drinking water produced renal cortical adenocarcinomas, some of them metastatic to the lung, liver, and spleen (*70*). These tumors occurred only in coexposed rats. A growing body of evidence indicates that nickel enhances the cytotoxicity and genotoxicity of DNA-damaging agents through inhibition of DNA repair. Thus, exposure to particulate black NiO and soluble $NiCl_2$ affected the removal of DNA adducts formed by benzo[*a*]pyrene in human lung cells (*69, 71*). Nickel inhibits the repair of *O*6-methylguanine and *N*7-methylguanine induced by treatment with *N*-methyl-*N*-nitrosourea in Chinese hamster ovary cells (*72*). Nickel blocks the removal of cyclobutane pyrimidine dimers produced by UV light exposure in HeLa cells (*73*).

More recently, a new class of DNA repair enzymes has been found. It includes alkyl DNA dioxygenases, such as the *Escherichia coli* AlkB dioxygenase and its two human homologues, ABH2 and ABH3, which facilitate a novel mechanism of DNA repair (*74*). In the presence of oxygen, these enzymes can specifically hydroxylate alkyl groups on 1-methyladenine and 3-methylcytosine (*75*). The requirement of iron for the reaction as well as inhibition of hydroxylase activity in crude cell extracts by iron chelators suggested that these enzymes are iron- and ascorbate-dependent. The activity of these enzymes, similar to protein hydroxylases, depends on iron oxidation status and may be inhibited by nickel exposure. These results indicate that the nucleotide and base excision repair pathway is affected by water-soluble and particulate nickel compounds and provide further evidence that DNA repair inhibition may be one of the mechanisms involved in nickel cocarcinogenic activity.

In addition to the inhibition of DNA repair, epigenetic changes induced by nickel compounds should be taken into consideration. The induction of cytosine methylation and histone deacetylation may lead to the heritable inactivation of the expression of senescence/tumor suppressor gene(s) and additionally contribute to the carcinogenic mechanism (*29, 30*). It is noteworthy that in animals nickel-induced carcinogenesis is known to be tissue, strain, and species-dependent (*1*). This suggests that genetic predispositions, including variations in the expression of genes involved in the metabolism of antioxidants, most likely glutathione and vitamin C, in different species and strains of animals, may also play an important role in nickel carcinogenesis (*76*). It is conceivable that similar genetic predispositions take place in human populations.

In conclusion, carcinogenic nickel produces significant alterations in cellular metabolism, which include, but are not limited to stimulation of glycolytic activity, alteration of iron homeostasis, depletion of ascorbate, and hypoxic stress. These effects of nickel exposure are summarized in Figure 1. It is clear that such metabolic alterations will lead to the modulation of gene expression through epigenetic changes. A coexposure with genotoxic carcinogens may exacerbate nickel effects.

3. Arsenic

3.1. Human Exposure and Carcinogenicity. Arsenic is an environmental contaminant, which can be found in the soil, water, and airborne particles as the result of both natural and human activities (*77, 78*). Epidemiological studies have confirmed that exposure to arsenic and its compounds can have adverse effects on human health. Inhaling arsenic can cause lung carcinomas, while ingestion in food or water, can provoke skin, respiratory system, liver and bladder tumors as well as diabetes and cardiovascular and neurological diseases (*77*). Humans are clearly more sensitive to inorganic arsenic carcinogenesis than animals. In rodents, it has proven difficult to induce tumors after inorganic arsenic exposure alone, making it problematic, in the absence of an animal model, to study mechanisms of arsenic carcinogenesis (*79*). Exposure to arsenic occurs generally in the form of either arsenite (As^{III}) or arsenate (As^{V}). The increase in cancer risk observed in epidemiological studies is attributed mainly to the exposure to inorganic arsenite, which is more toxic than arsenate (*80, 81*). This may be due to a better cellular uptake of arsenite, which, at equimolar concentration, is accumulated in many cell types much faster as compared with arsenate. Inside the cell, arsenate may be reduced to arsenite. Glutathione seems to play an important role in arsenate reduction and detoxification through conjugation (*82*) (Figure 2). After

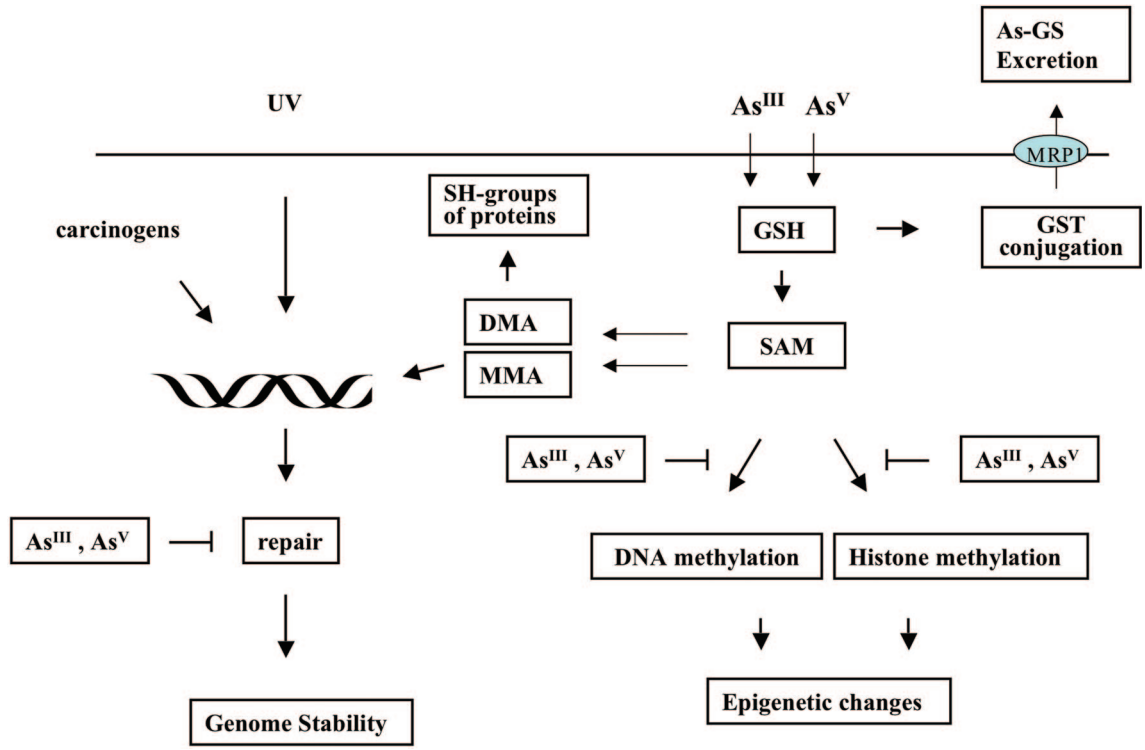

Figure 2. Schematic representation of major cellular interactions of tri- and pentavalent arsenic. Both forms, tri- or pentavalent arsenic, can enter cells. Inside the cells, pentavalent arsenate can be reduced by glutathione to trivalent arsenite, and/or GSH-As can be methylated to pentavalent or trivalent monomethylated (MMA) or dimethylated (DMA) arsenic. DMA^{III} and MMA^{III} could react with sulfhydryl groups of some proteins. This will result in modification of protein function and retention of arsenic inside the cell. Arsenic methylation can deplete *S*-adenosylmethionine, which serves as a universal donor of the methyl group. As a result, DNA or histone methylation pattern may be changed. GSH-As complexes can be excreted from cells by the MRP1 transporter. MMA or DMA along with other carcinogens or with UV radiation can induce DNA damage. The repair of this damage may also be inhibited by arsenic.

ingestion, arsenic is rapidly excreted, primarily through urine, mainly in the form methylated arsenic metabolites (*83*). These methylated metabolites are produced *in vivo* by conjugation using *S*-adenosylmethionine as the methyl donor (*82, 84*). The methyltransferases involved in the process are not yet characterized. Methylated arsenic metabolites include pentavalent or trivalent monomethylated (MMA) and dimethylated (DMA). The levels of *S*-adenosylmethionine are important in arsenic metabolism since a low intake of dietary methyl groups, that is, low dietary content of methionine or folate deficiency, results in lower arsenic methylation (*85*). Since methylated species are excreted much faster than inorganic arsenic species, it is conceivable that methylation represents a part of detoxification program.

Pentavalent arsenicals were not shown to be a carcinogenic risk to humans at typical environmental exposures. However, dimethylarsinic acid (DMA^{V}) is carcinogenic at high doses to the rat urinary bladder but not in mice (*86*). The carcinogenic mode of action involves cytotoxicity followed by regenerative cell proliferation. The cytotoxicity is due to the formation of a reactive metabolite, most likely dimethylarsinous acid (DMA^{III}), which causes oxidative damage and/or binds with critical urothelial sulfhydryl groups (*86*). Thus, these data indicate that methylated forms of arsenic may be potentially more dangerous than nonmethylated ones. This, however, will require further investigations. It is noteworthy, that arsenicals administered to the rat may also bind to a specific cysteine in the hemoglobin alpha chain as $DMA(^{III})$, regardless of the arsenical being administered. Hemoglobin binding is responsible for the greater accumulation of arsenic species in rat blood than in human or mouse blood. The significance of this modification is not clear; however, it is conceivable that this may be the reason that the rat is one of the few arsenic carcinogenic animal models available at present.

3.2. Increased Cellular Proliferation. In cell and animal models, arsenite and perhaps some methylated metabolites may activate signal transduction pathways, which enhance cell proliferation, reduce antiproliferative signaling or inhibit differentiation, and override checkpoints controlling cell division after genotoxic insult (*87, 88*). In animals treated with arsenite, hyperplasia is seen in the urinary bladder epithelium and in skin (*89, 90*). In human keratinocytes, increased mRNA transcripts and secretion of keratinocyte growth factors, including granulocyte macrophage-colony-stimulating factor (GM-CSF) and transforming growth factor-α (TGFα), were observed (*91*). In a human uroepithelial cell line, arsenic activates EGFR and ERK in a ligand-independent manner, which does not involve autophosphorylation Tyr(1173) (*92*). c-Src activity is also induced by arsenic and is a prerequisite for the EGFR and ERK activation. Activation of growth factor receptors regulates G_1 phase cyclins and associated cyclin-dependent kinases (cdks). Cyclin D1 is one of the cyclins up-regulated by arsenite exposure (*93, 94*). Cyclin D1 has a very short half-life, and its protein levels depend on phosphorylation by a number of kinases, including extracellular signal-related kinases (ERKs), phosphotidylinositol 3-kinase (PI3K), and IKK. Initially, in human fibroblasts, it was found that long-term and low-dose (but not short-term, high-dose) exposure to arsenite resulted in increased expression of cyclin D1 (*93*). However, it was later shown that even 12 h of exposure to low-dose arsenite caused cyclin D1 up-regulation in human keratinocytes (*94*). Low concentrations of arsenite also disrupt p53 function, which results in down-regulation of p21 in response to genotoxic stress (*93*). Murine fibroblasts chronically exposed to low concentrations of arsenite

show increased proliferative response to epidermal growth factor (EGF) and increased expression of c-myc and E2F-1 (positive growth regulators) (*95*). Thus, exposure to arsenite results in the stimulation of cell-cycle progression, especially G_1-S transition. Down-regulation of p53 function suggests that in the presence of arsenic cells may enter the cell cycle with unrepaired DNA lesions.

3.3. Apoptotic Effects of Arsenite Exposure and NF-kB Signaling Pathway. Arsenic trioxide is an effective treatment for acute promyelocytic leukemia (APL) (*96*). The APL patients resistant to all-trans retinoic acid and other types of chemotherapy can still respond to arsenic trioxide. *In vitro* studies showed that at micromolar concentration arsenic trioxide triggers APL cell apoptosis. Lower doses of arsenic were shown to induce differentiation of leukemic cells. Both apoptosis and cell differentiation are important factors of arsenic therapeutic effect. Another type of malignancy, which is often resistant to chemotherapy, is adult T-cell leukemia/lymphoma (ATL). Recent data showed that combined exposure of ATL cells to arsenic and IFN-α has dramatic synergistic effects on both cell cycle arrest and induction of apoptosis in these cells (*97*). The apoptotic effect of arsenic was caused by an up-regulation of IkB-α, resulting in a sharp decrease in DNA binding of NF-kB complexes and suppression of NF-kB target genes due to the cytoplasmic retention of RelA (*97*). Similar to the effect on lymphocytes, sodium arsenite downregulates NF-kB activity by inhibiting phosphorylation and subsequent degradation of IkB-α in Caco-2 cells (*98*). The stabilization of IkB-α by sodium arsenite did not require reactive oxygen species formation (*98*). Thus, therapeutic effect of arsenic is mediated through down-regulation of an important antiapoptotic transcription factor NF-kB. The effect of arsenite on NF-kB was extensively studied *in vitro*. The experimental findings, however, are contradictory. In some instances, the exposure to arsenite up-regulated NF-kB (*99–101*). In others, the exposure to arsenite down-regulated NF-kB (*102, 103*). This controversy could be explained by the use of different cell models as well as by different time and doses of treatment. Thus, low-dose and short-term of treatment with sodium arsenite could activate NF-kB DNA binding, whereas chronic exposure to 0.1 or 0.5 μM As(III) decreased NF-kB DNA binding activity (*104*).

3.4. Genetic and Epigenetic Changes. Arsenic is known to induce deletion mutations and chromosomal alterations, such as aberrations, aneuploidy, and sister-chromatid exchanges, but not point mutations (*105*). In spite of its low mutagenic activity, arsenic has high transforming activity (*106*). Among genetic changes, it is also important to note that arsenic exposure can cause gene amplification. Thus, two arsenic salts, sodium arsenite and sodium arsenate, were shown to induce a high frequency of methotrexate-resistant 3T6 cells (*107*). The resistance was due to the amplification of the dihydrofolate reductase gene. The ability of arsenic to induce gene amplification may relate to its carcinogenic effects in humans since amplification of oncogenes is observed in many human tumors. A large body of evidence has been accumulated over the last 10 years suggesting that epigenetic changes are important in arsenic carcinogenesis. Thus, exposure to arsenic can induce both DNA hypomethylation and hypermethylation. DNA methylation changes are typically observed in cancer, in which global methylation is reduced, but some gene-specific promoter methylation is increased (*108*). The exposure of human lung adenocarcinoma A549 cells to arsenite results in increased cytosine methylation in the p53 promoter (*109*). Both hypo and hypermethylation of different genes was found in human kidney cells treated with arsenite *in vitro* (*110*). Global DNA hypomethylation and evidence of depressed levels of *S*-adenosylmethionine and decreased DNA methyltransferase activity were found in rat liver epithelial cell line following chronic exposure to low levels of arsenic (*111*). Chronic oral exposure of A/J mice to inorganic arsenate, which results in the appearance of lung tumors, causes a decrease or loss of $p16^{INK4a}$ and RASSF1A expression in these tumors as compared to that in nontumor lung tissues from both control and inorganic As^V-exposed mice (*112*). This reduced or lost expression was the result of hypermethylation of these genes. Significant DNA hypermethylation of the promoter region of p53 gene was observed in the DNA of arsenic-exposed humans compared to that in control subjects (*113*). This hypermethylation showed a dose–response relationship. Furthermore, hypermethylation of the p53 gene was also observed in arsenic-induced skin cancer patients compared to that in subjects having skin cancer unrelated to arsenic, though not at a significant level. However, a small subgroup of cases showed hypomethylation with high arsenic exposure. Significant hypermethylation of the gene p16 was also observed in cases of arsenicosis caused by high levels of arsenic. Thus, the ability of arsenic to alter DNA methylation in humans may be important in carcinogenesis. Unlike nickel, exposure to arsenite does not cause methylation and silencing of the transgenic *E. coli gpt* gene in Chinese hamster G12 cells, which indicates that these two carcinogenic metals are acting through different pathways (*29*).

3.5. Metabolic Changes. Arsenite was shown to inhibit pyruvate dehydrogenase (PD) activity through binding to vicinal dithiols in pure enzyme and tissue extract. However, more recently, it was shown that arsenite may cause oxidation and inactivation of PD by ROS, which inactivates an enzyme (*114*). This can occur at a much lower concentration than that needed for direct binding of arsenite to the critical thiols. The ROS produced by arsenite may up-regulate HIF-1 and downstream VEGF in normoxic H134 and OVCAR-3 cells (*115*). Pretreatment with the ROS inhibitors catalase and mannitol attenuated arsenite-induced ROS production but did not affect induction of VEGF mRNA and HIF-1α protein. In contrast, pretreatment with the thiol antioxidants glutathione or *N*-acetylcysteine completely abrogated both effects, whereas a potentiation was observed by the depletion of intracellular glutathione. Further studies, however, did not confirm transcriptional up-regulation of hypoxic genes. Thus, sodium arsenite did not activate a HIF-1-dependent reporter gene in OVCAR-3 cells, indicating that functional HIF-1 was not induced. In agreement with this hypothesis, up-regulation of VEGF mRNA was not reduced in HIF-1α(–/–) mouse fibroblast cell lines. Altogether, these data suggest that not HIF-1 but rather p38 mediates the induction of VEGF mRNA expression by sodium arsenite (*116*).

Arsenic reduction and methylation is aimed to detoxify and excrete this toxic metalloid from cells. Indeed in human urine, the major metabolites of inorganic arsenicals, such as arsenite and arsenate, are MMA^V and DMA^V. However, in rat bile, the major metabolites of inorganic As^{III} have been reported to be arsenic–glutathione (As-GSH) complexes. As-GSH complexes can be transported from cells via the multidrug resistance-associated protein (MRP) family (*117, 118*) (Figure 2). Consistent with this notion, C57BL/6 mice, which have a higher expression level of MRP1, but not other ABC transporters, were shown to be more resistant than BALB/c mice to sodium arsenic induced renal injury (*119*). The complex relationship between glutathionylation and methylation of arsenic was investigated recently. It was suggested that As-GSH complexes are substrates

for arsenic methyltransferase; therefore, all observed products are components of the arsenic metabolic pathway (*82, 120*).

3.6. Arsenic as a Cocarcinogen. At low nonmutagenic concentrations, arsenite can enhance the mutagenicity of other carcinogens, probably by interfering with DNA repair (*79*). Arsenite enhances the mutagenicity and/or clastogenicity of UV, *N*-methyl-*N*-nitrosourea (MNU), diepoxybutane, X-rays, and methylmethane sulfonate in mammalian cells (*121–124*). Arsenic inhibits the repair of DNA adducts caused by benzopyrene in rats (*125*). In mice, arsenite can enhance the carcinogenicity of ultraviolet radiation (UV) (*126, 127*). Combined exposure of 10 mg/L sodium arsenite in drinking water with 1.7 KJ/m^2 solar UVR 3 times weekly after 26 weeks resulted in a 2.4-fold increase in skin tumor yield compared with mice given UV alone. No tumors appeared in any organs in control mice or in mice given arsenite alone. The molecular mechanism for tumor formation involves reduction in the repair rate of photoadducts and inhibition of apoptosis (*128, 129*).

Recently, it has been shown that a short period of maternal exposure to inorganic arsenic in the drinking water results in multitissue carcinogenesis in the adult offspring (*130*). For example, prenatally exposed female C3H offspring showed dose-related increases in ovarian tumors and lung carcinoma and in proliferative lesions (tumors plus preneoplastic hyperplasia) of the uterus and oviduct. In addition, prenatal arsenic plus postnatal exposure to the tumor promoter, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) in C3H mice produced excess lung tumors in both sexes and liver tumors in females. In CD1 mice, additional postnatal treatment with diethylstilbestrol or tamoxifen after prenatal arsenic exposure induces urinary bladder transitional cell proliferative lesions, including carcinoma and papilloma, and enhances the carcinogenic response in the liver of both sexes. These data provide convincing evidence that arsenic is a transplacental carcinogen and cocarcinogen in mice with the ability to target tissues of potential human relevance, such as the urinary bladder, lung, and liver.

In conclusion, arsenic is acting as a nongenotoxic carcinogen mainly via alterations in DNA methylation (Figure 2). It cannot be excluded that arsenic may also cause alterations in histone methylation, although this has not been shown yet. Because of its inhibitory effects on DNA repair, arsenic acts as a very efficient cocarcinogen.

4. Chromium

4.1. Human Exposure and Carcinogenicity. Cr and its compounds have a long history of industrial uses in the manufacture of a large number of high-volume products, such as stainless steel and pressure-treated wood. Occupational exposure to Cr is found among approximately half a million industrial workers in the United States and several million worldwide (*131, 132*). Environmental exposure likely impacts millions of people drinking Cr-containing water, residing in the vicinity of numerous toxic sites, and various industrial users of Cr products. One example of widespread exposure is the presence of significant contamination with hexavalent Cr in approximately 30% of the drinking water sources in California[1]. The presence of Cr in urban particulate matter and emissions from automobile catalytic converters leads to exposure by very large segments of populations in densely populated areas.

Although Cr can exist in several valence states, the most commonly encountered products contain this metal in the +6, +3, and 0 oxidative forms (*133*). Cr(0) is usually present in its metallic form, which typically occurs in alloys with other metals, particularly Fe and Co. Welding and other strongly oxidizing conditions convert Cr(0) to Cr(III) and Cr(VI). Cr(III) is thermodynamically stable, and it is the final oxidative form found in all biological systems. Depending on the nature of the counter ion, the solubility of Cr(VI) compounds varies from very high (salts with alkali metals) to moderate (salts of Ca, Mg, Sr, and Zn) to very low (barium and lead salts). The highest exposure to Cr(VI) occurs in chromate manufacturing, chrome plating, ferrochrome production, and stainless steel welding. Welders employed in construction and small car repair shops are at particular risk of heavy exposure because of the absence or practical difficulties in the installation of exhaust systems for removal of Cr(VI)-containing fumes from the breathing area.

Occupational exposure to Cr(VI) compounds but not other oxidative forms of Cr is a well-documented cause of respiratory cancers (*21, 134, 135*). Cr(VI)-associated neoplasms are typically located in the lung, but risk of nasal cancers is also significantly increased (*136–138*). Contrary to some very optimistic views that Cr(VI) carcinogenesis is caused only by massive exposures and that therefore it is no longer a concern (*139*), recent epidemiological and risk-assessment studies have actually found as much as 25% lifetime risk of dying of lung cancer under 52 μg/m^3 permissible exposure limit (*135, 140*). This standard originally adapted by OSHA in 1971 was lowered 10-fold to 5 μg/m^3 in 2006 (*132*), but even the new standard is expected to result in an additional 10–45 deaths per 1000 exposed workers. In parallel with epidemiological findings, recent studies in cells cultured under more biologically relevant conditions found a much greater potential for Cr(VI) to cause chromosomal damage and mutations (*141*) than it was previously thought.

Although a frequently referenced review by IARC in 1990 (*21*) found stronger evidence for the carcinogenicity of less soluble chromates, specifically for salts with moderate solubility, the follow-up epidemiological studies clearly showed that human exposure to soluble chromates also significantly increased the risk of lung cancer (*142, 143*). The ability of all types of cells to take up, metabolize, and form genotoxic Cr-DNA damage from Cr(VI) (section 4.2) lends major mechanistic support for the view that Cr(VI) can induce malignancies at sites outside the respiratory system (*144, 145*). A recently released draft of the NTP report[2] on testing of dichromate in drinking water contains clear evidence of its carcinogenicity in the oral cavity and small intestine. While NTP studies have not found evidence for systemic cancers following the ingestion of dichromate, exposed animals showed clear signs of toxicity in the liver and other internal organs, indicating the ability of toxic Cr(VI) to avoid detoxification in the GI tract and enter systemic circulation. Sustained elevation of Cr levels in red blood cells of human volunteers following ingestion of Cr(VI)-laced water (*146*) is also consistent with the absorption of significant amounts of Cr(VI) into the blood (*131*).

Insoluble salts of lead and barium chromates were negative in the implantation model of lung carcinogenesis (*147*), and epidemiological data for their carcinogenicity are also not as strong as those for other chromates (*134*). However, considering that soluble chromates were also negative in the implantation model but are now confirmed as carcinogenic, it is prudent to consider all chromates as equally carcinogenic.

[1] www.dhs.ca.gov/ps/ddwem/chemicals/chromium6/samplingresults.htm

[2] NTP Technical Report on the toxicology and carcinogenesis studies of sodium dichromate dihydrate in F344/N rats and B6C3F1 mice. NIH Publication # 07–5887, 2007.

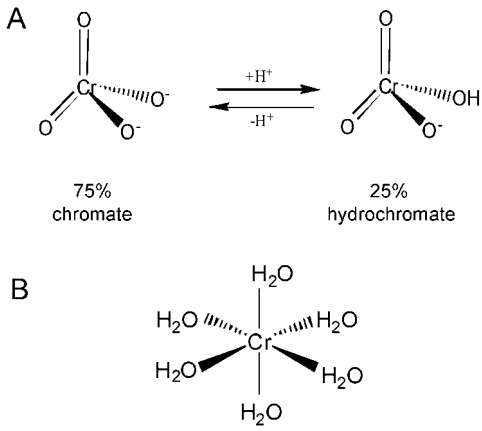

Figure 3. Structures of tetrahedral Cr(VI) and octahedral Cr(III) complexes. (A) Chromate (CrO_4^{2-}) and hydrochromate ($HCrO_4^-$) are the main aqueous forms of Cr(VI) at neutral pH. (B) Octahedral arrangement of H_2O groups in hexacoordinate complexes of Cr(III). At neutral pH, the $Cr(H_2O)_6^{3+}$ complex undergoes rapid hydrolysis, producing a mixture of mononuclear and polynuclear species containing hydroxo ligands (*133*).

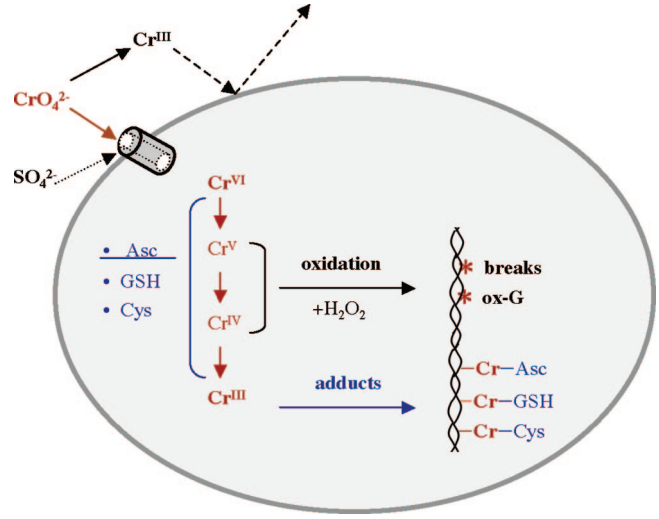

Figure 4. Major steps in uptake, metabolism, and formation of DNA damage by Cr(VI).

Squamous cell carcinoma is the most common form of lung malignancies in chromate workers, and the majority of tumors were located in the central part of the lung (*148*). Interestingly, Cr(VI) exposure was associated with the development of multiple tumors in several subjects, pointing to the potential existence of individual susceptibility factors in Cr-induced carcinogenesis. Squamous cell carcinoma appears to develop from dysplastic lesions over a relatively short period of about one year (*149*). The location of lung tumors corresponds to the sites of Cr accumulation, with bronchial bifurcations exhibiting the highest Cr levels in ex-workers who had been exposed to soluble chromates (*150*). Long-term retention of Cr in the bronchial tissue primarily occurred in the stroma (*151*), probably reflecting a faster clearance of Cr from the more rapidly renewable bronchial epithelia. Sedimentation and inertial impaction of typical chromate particles with 1–3 μm in diameter were likely the main reasons for the high deposition of Cr in areas of bifurcation. Suppressed mucus flow, delaying effective clearance of chromate particles, could be another contributing factor since chronic exposure to respiratory toxicants causes the loss of cilia near bifurcations (*150*). Lung-deposition profiles of Cr from particles of lower solubility chromates are currently unknown, but they are expected to show the same pattern of hotspots. Although the majority of lung cancers were found among chromate workers who smoked (*135, 148*), this may simply reflect a fact that the majority of workers were smokers. Smoking does not affect Cr accumulation in the lung (*148*), and chromate exposure was clearly established as an independent risk factor for lung cancer (*135*). Molecular features of chromate and smoking-associated cancers are very different (section 4.4), arguing for the smoking-unrelated origin of the majority of chromate malignancies.

4.2. Cr(VI) Metabolism and DNA Damage. At neutral pH, Cr(VI) exists as a mixture of chromate (CrO_4^{2-}) or hydrochromate ($HCrO_4^-$) anions with the approximate ratio of 3:1 (*133*) (Figure 3). Chromates are isostructural with physiological sulfate and phosphate ions, and because of this molecular mimicry, Cr(VI) readily enters cells through the sulfate channels (*152*). Human and other mammalian cells are capable of massive accumulation of Cr(VI), with cellular levels 10–20 times above those outside the cell within 3 h (*141, 153*). A 24-h long incubation can lead to 100-fold or higher accumulations (*154*). Cr(VI) is a pro-carcinogen that by itself is completely unreactive toward DNA under physiological pH and temperature. In the biological systems, however, Cr(VI) undergoes a series of reduction reactions yielding thermodynamically stable Cr(III). When this occurs extracellularly, reduction acts as the detoxification process because of the production of poorly permeable Cr(III) complexes (Figure 4). Inside the cell, Cr(VI) reduction is the activation event that is responsible for the generation of genotoxic damage and other forms of toxicity. Unlike the majority of other human pro-carcinogens, Cr(VI) metabolism in mammalian cells does not require any enzymes and relies on the direct electron transfer from ascorbate and nonprotein thiols, such as glutathione and cysteine (*133*). Studies of chromate reduction in tissue homogenates (*155–157*) and measurements of reduction rates in defined reaction mixtures (*158, 159*) all showed that ascorbate was the dominant biological reducer of Cr(VI), accounting for about 90% of its metabolism in cells *in vivo*. In contrast to the millimolar levels of ascorbate in cells *in vivo* (e.g., 1.3 mM in human lung; ref *160*), cultured human and nonhepatic cells from other species contained at best about 50–60 μM but usually much lower ascorbate concentrations (*61, 141, 153, 159*), and they rely on thiols for Cr(VI) reduction (*133*). Thus, unless cellular ascorbate levels are restored to normal, typical cell cultures provide a nonphysiological model of Cr(VI) metabolism, which has recently been found to underestimate the genotoxic and mutagenic abilities of Cr(VI) (*141*). Even delivery of as low as 80 μM ascorbate into A549 cells was found to have a strong effect on the yield of intermediate reaction products (*161*).

The end-product of Cr(VI) metabolism in all biological systems is always Cr(III) (*162–164*), but the reduction process can also generate variable amounts of Cr(V), Cr(IV), and organic radicals depending on the reducer and the ratio of reactants (*165–167*). Under conditions of ≥2-fold molar excess of the reducer, reactions of Cr(VI) with ascorbate generate Cr(IV) as the first Cr intermediate (*166–168*). The presence of Cr(V) was only detectable when high concentrations of the reactants were used and ascorbate was present at nonphysiological 1:1 or lower ratio to Cr(VI). The formation of Cr(V) in the mixtures containing ascorbate concentrations that were insufficient to complete Cr(VI) reduction probably resulted from the secondary reactions of Cr(IV) (CrVI + CrIV → 2CrV and 2CrIV → CrV + CrIII). While the presence of small amounts of short-lived Cr(V) at higher than 2-fold ratio of ascorbate to Cr(VI) cannot be excluded because of the technical limitations of the employed

spin resonance spectroscopy approaches, it is doubtful that environmental levels of Cr(VI) will be sufficient to produce significant quantities of Cr(V) in cells with millimolar ascorbate concentrations. The first step in the reduction of Cr(VI) by physiological concentrations of cysteine proceeds primarily through one electron transfer (*169*), which explains a strong signal for Cr(V) in Cr(VI)-cysteine reactions (*170*). The initial electron transfer reaction in the glutathione-driven reductions is predominanatly a two-electron process (*171*), but the production of Cr(V)-glutathione species is also readily detectable (*165, 170, 172*). The final product of Cr(VI) metabolism, Cr(III), forms stable coordination complexes with nucleic acids and proteins. *In vitro* reduction reactions also generate large amounts of Cr(III) complexes containing two molecules of the unoxidized reducer (*173, 174*). Studies in Cr(VI)-treated cells and *in vitro* reduction reactions produced evidence for the formation of several types of DNA damage, including strand breaks and various Cr-DNA adducts.

4.2.1. Cr-DNA Adducts. Small Cr-DNA adducts are the most abundant form of Cr(VI)-induced genetic lesions in mammalian cells (*133*), and they were found to be responsible for all mutagenic damage generated during Cr(VI) reduction with cysteine (*175*) and ascorbate (*158*). The majority (50–75%) of adducts generated during *in vitro* Cr(VI) reductions are binary Cr-DNA complexes (*159, 176*). Binary adducts are only weakly mutagenic (*158, 177*), and their existence in cells is uncertain because of the presence of numerous Cr(III)-binding small molecules. Ternary Cr-DNA adducts can be disrupted during DNA isolation (*159*), which produces binary adducts and further complicates the assessment of the real levels of these small adducts. The predominant form of Cr-DNA complexes in cells are ternary adducts (cross-links), which include Cr(III) atom bridging DNA and small cellular molecules (L-Cr-DNA). Four major forms of ternary adducts are glutathione-Cr-DNA, cysteine-Cr-DNA, histidine-Cr-DNA, and ascorbate-Cr-DNA complexes (*159, 178, 179*). All ternary adducts were much more mutagenic than binary adducts, and ascorbate-Cr-DNA cross-links were the most potent premutagenic Cr-DNA modifications (*158, 177*). Ternary adducts are formed through an attack of DNA by preformed ligand-Cr(III) complexes (*180*). Binary adducts can also be generated in the direct reaction of newly formed Cr(III) with DNA (*175, 176, 180*), but the possibility that a fraction of binary adducts results from the reaction of intermediate Cr forms, particularly Cr(IV), cannot be excluded. Cr(V) complexes exhibit little or no direct binding to DNA (*181, 182*), and the presence of Cr(V) in Cr(VI) reduction reactions is not required for the formation of Cr-DNA adducts *in vitro* (*158, 159*) or in cells (*141, 153*). The primary site of attachment for all Cr(III) adducts is the phosphate group (*180, 183*), but induction of G/C-targeted mutagenic events by Cr-DNA modifications (*175, 177*) has also led to the suggestion that the mutagenic forms of adducts are probably Cr(III) microchelates involving a phosphate group and the N^7 position of G (*175*). Thus, there are apparently two classes of Cr-DNA phosphate adducts: the majority (about 90%) are nonmutagenic monofunctional Cr-phosphate complexes, while the minority are mutagenic phosphate-N^7dG microchelates. Both types of adducts are substrates for nucleotide excision repair (NER) in human (*184*) and hamster (*185*) cells, as evidenced by the persistence of total adducts and increased toxicity and mutagenicity of Cr-DNA damage in NER-deficient cells. A recent assignment of Cr-dG binding to NGG sequences (*186*), which was based on the mapping of DNA nicks made by bacterial UvrABC exinuclease in Cr-adducted DNA, is consistent with the sequence specificity of Cr-adduct mutagenesis (*177*). The discrepancy in the reported nucleotide specificity of Cr-DNA binding determined by UvrABC mapping (*186*) and other approaches (*177, 180*) can be related to the ability of UvrABC to recognize only a fraction of DNA adducts with the largest degree of duplex distortion. Preferential binding of Cr(III) to the phosphate backbone leads to only minor 1–2° distortions in the DNA duplexes (*187*).

4.2.2. DNA–Protein and DNA Interstrand Cross-Links. The formation of DNA–protein cross-links (DPC) by Cr(VI) is well established in various biological systems (*188, 189*) and in the *in vitro* reactions (*190*). The overall yield of DPC in cells was estimated to be less than 1% of all Cr-DNA adducts, but it could be significantly higher *in vitro* (*133*). The availability of sensitive methodologies led to the frequent use of DPC measurements as a biomarker of Cr(VI) exposure in humans (*190, 192*) and aquatic species (*193*). The biological significance and repair of Cr-induced DPC remain largely unknown; however, a very large size of these lesions would likely represent a major obstacle for the replication and transcription processes. The presence of interstrand DNA cross-links have been detected only under certain *in vitro* conditions (*176, 194, 195*), and on the basis of the severe steric restrictions for the intercalation of octahedral Cr(III) complexes, it was argued that interstrand cross-links were probably produced by Cr(III) oligomers (*133*). The presence of oligomeric Cr(III) forms is very unlikely inside the nucleus and the lack of Cr(VI) hypersensitivity in cross-link repair-deficient ERCC4(XPF)-null CHO cells (*185*) provided a strong argument that DNA cross-linking is probably an *in vitro* phenomenon arising under conditions of high Cr(III) and low ligand concentrations. If formed, the interstrand cross-link would represent a potent block for cellular DNA replication.

4.2.3. DNA Breaks. The presence of single-strand breaks (SSB) in chromate-treated cells in culture and in animal tissues has been reported in several studies (*196–199*) that used standard detection assays for the quantitation of these DNA lesions. While the results of these and related studies showed clear positive responses, the reliance of all of the employed methodologies (alkali elution, alkaline unwinding assay, and alkaline single cell electrophoresis) on DNA unwinding in strongly alkali conditions raises a major concern as to whether the recorded data measured genuine SSB or breaks that were artifacts of the alkaline assay conditions. Cr-DNA phosphate adducts (*176*), similar to other modifications of DNA phosphate groups, make phosphodiester linkages unstable under alkaline conditions, causing breaks. In fact, this property of alkylphosphotriesters has long been used for their quantitation in cellular DNA (*200*). Another complication in the detection of SSB is a rapid excision of Cr-DNA adducts by NER (*184*), which in human cells generates about 50,000 excision events/min following exposure to 2–5 μM Cr(VI). Assessment of the formation of toxic SSB by genetic approaches relying on the comparison of Cr(VI) toxicity in SSB-repair deficient (*XRCC1–/–*) and proficient (*XRCC1+*) isogenic EM9-CHO cells (*154*) showed that toxic SSB were formed at Cr(VI) doses, causing more than 50% clonogenic lethality and accumulation >0.5 mM cellular Cr. The production of SSB was inhibited by the addition of catalase and iron chelators or by elevation of glutathione levels. These results along with the oxygen dependence of SSB induction in glutathione–chromate mixtures (*201*) led to the suggestion that SSB were caused by oxidizing species generated in the reaction of Cr(V) with H_2O_2 (*154*). The ability of Cr(V) to act as a catalyst in Fenton-like reactions with H_2O_2 has been well established (*181, 202*). The source of H_2O_2 *in vitro* has been

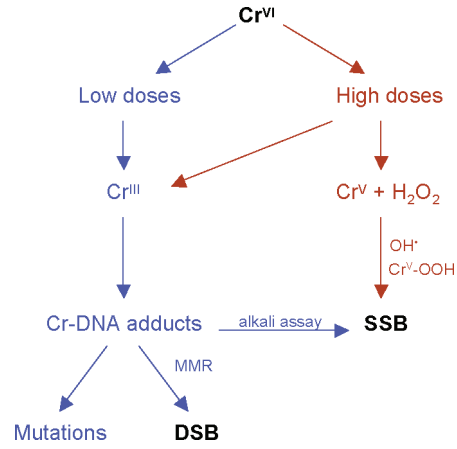

Figure 5. Direct and indirect mechanisms in the generation of single-strand (SSB) and double-strand (DSB) DNA breaks by Cr(VI).

traced to Fe contamination, particularly in the stock preparations of the reducers (*154, 158, 176*). In Cr-treated cells, increased production of H_2O_2 could be a result of mitochondrial damage or elevated activity of NADPH oxidases. Since ascorbate-driven reductions generate minimal if any Cr(V) at biologically relevant conditions (*166–168*), then the formation of SSB in ascorbate-supplemented cells should be very low. In support of this suggestion, *in vitro* reactions of Cr(VI) with 0.2 and 1 mM ascorbate concentrations found no significant DNA strand breakage (*158, 203*), but experiments in human cells with restored physiological levels of ascorbate are certainly needed to directly examine this question.

Cytogenetic studies in Cr(VI)-exposed cells in culture and *in vivo* have long reported findings, such as increased frequencies of chromosomal breaks (*204, 205*) and micronuclei (*206*), consistent with the induction of DNA double-strand breaks (DSB). However, it is only very recently that more direct evidence for the formation of DSB in Cr(VI)-treated human cells has been obtained (*141, 207, 208*). DSB were produced via an indirect mechanism, which required the passage of cells through S-phase and the participation of mismatch repair proteins. A more detailed discussion of this phenomenon is presented in section 4.3. Figure 5 summarizes the major pathways leading to the production of SSB and DSB by Cr(VI).

4.2.4. DNA Base Damage. Damage to the base component of DNA can involve either loss of bases (i.e., production of abasic sites) or chemical modifications with the retention of altered bases in DNA duplexes. *In vitro* studies of Cr(VI) reductions with ascorbate and glutathione showed that the formation of abasic sites closely mirrored the yield of SSB and required the same reactive species (*201, 209*). Similarly to SSB, when iron-free reaction conditions were used, no abasic sites were observed (*158, 176*). Given the parallel production of both types of backbone damage, cellular conditions permitting the induction of SSB should also lead to the concomitant production of abasic sites. No direct measurements of abasic sites in Cr(VI)-treated human cells have yet been done.

Administration of Cr(VI) to animals with different tissue levels of ascorbate failed to induce the formation of 8-oxoG (*210*), which is the most widely used indicator of the oxidative insult on DNA. Similarly, GC/MS analyses of DNA modified in the presence of Cr(VI) and ascorbate or glutathione have not found 8-oxoG or other base oxidation products (*211*). Consistent with the absence of oxidative base damage, replication of Cr(VI)/reducer-treated plasmids in human cells generated no mutagenic events when Cr(III)-DNA binding was prevented or disrupted (*158, 175, 203*). However, Slade et al. (*212*) have recently reported that Cr(VI) reduction by ascorbate yielded significant amounts of spiroiminodihydantoin, which is one of the advanced oxidation products of guanine. Thus, it appears that guanine oxidation can occur during Cr(VI) reduction, but its main product was different from that typically expected for the oxidant-producing reactions. Apart from potentially significant differences in the reaction conditions, the discrepancy between the lack of mutagenic responses in shuttle-vector plasmids treated with Cr(VI)-ascorbate in phosphate buffer (*158, 203*) and the production of spiroiminodihydantoin in PBS buffer (*212*) may be related to either a weak mutagenicity of this lesion or its rapid repair in human cells. Determination of spiroiminodihydantoin and other advanced oxidation products of guanine in ascorbate-complemented human cells would be very useful to clarify their importance in genotoxic effects of Cr(VI).

4.3. Genomic Instability, Toxicity, and Cr(VI) Carcinogenesis. Cr(VI)-associated carcinogenesis differs from malignant processes involved in smoking-induced lung tumors by its very low frequency of mutational inactivation of p53 (*213*). A small number of p53 mutations found in chromate-induced lung cancers included base substitutions at A/T pairs and double missense mutations. This spectrum of mutagenic events is more consistent with the mutator phenotype of tumor cells since Cr(VI)-induced mutations do not occur at A/T bases pairs. In all biological systems examined to date, G/C pairs were the predominant targets of Cr(VI) mutagenesis (*158, 175, 177, 214, 215*). Thus, the Cr(VI)-activated malignant process proceeds through a completely different pathway despite that fact the majority of lung cancers were found among chromate workers who were smokers (*135*).

One interesting feature of Cr(VI)-associated cancers was the presence of microsatellite instability (*216*), which indicates a complete loss of functional mismatch repair (MMR) (*217, 218*). In Cr-induced cancers, microsatellite instability was associated with the loss of expression of MLH1 (*219*), which is one of the essential MMR proteins. The absence of MMR leads to the inability of cells to correct replication errors, and these cells exhibit 100-times higher mutation rates at their chromosomal genes, but the frequency of mutagenic events is even greater in the areas of simple nucleotide repeats known as microsatellites. Thus, chromate-associated cancer cells express the mutator phenotype caused by the loss of the major mutation avoidance system, MMR. Once cells inactivated MMR, the subsequent acquisition of mutations in the critical growth-controlling genes is greatly accelerated since these cells maintain high rates of random mutagenesis and no longer need continuous exposure to Cr(VI) for additional mutagenic events. Then the question arises as to how and why Cr(VI) selectively leads to the appearance of this specific form of genomic instability, which is uncommon for other lung carcinogens. The answer to this question appears to lie in the active role that MMR plays in toxic and genotoxic effects of Cr(VI).

MMR-deficient mouse and human cells have recently been found to be resistant to apoptosis and clonogenic lethality of Cr(VI) (*153, 208*). The absence of MMR also eliminated the ability of Cr-DNA adducts to inhibit cellular replication of Cr-modified vectors and strongly suppressed the induction of DSB in Cr(VI)-treated cells (*141, 208*). Thus, MMR acted as the aberrant repair process, generating genotoxic damage rather than eliminating it. The Cr-resistant phenotype was induced by the loss of any of the four main MMR proteins (PMS2, MLH1, MSH2, or MSH6) (*153, 208*), indicating that the entire MMR complex was required for the processing of Cr-DNA adducts

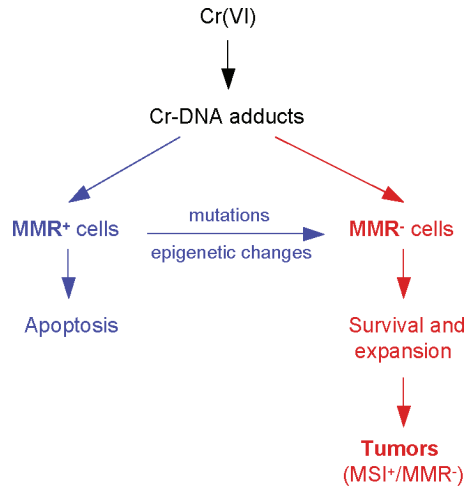

Figure 6. Selection model of Cr(VI) carcinogenesis. Resistance of mismatch repair-deficient (MMR^-) cells allows their survival and expansion during repetitive exposures to toxic doses of Cr(VI). MMR^- cells can arise spontaneously and can be caused by mutagenic Cr-DNA damage. Very high rates of spontaneous mutagenesis in MMR^- cells lead to accelerated acquisition of the necessary mutations in the critical cancer-controlling genes, and resulting tumors exhibit microsatellite instability (MSI^+ phenotype).

into highly toxic DSB. The damage-promoting effects of MMR extended to the full range of Cr(VI) concentrations from very low nontoxic (<1 μM) to highly toxic doses (>90% clonogenic lethality). The potentiating effects of MMR were most strongly pronounced in cells supplemented with physiological levels of ascorbate (*141, 153*). Apoptotic responses and clonogenic lethality induced by Cr(VI) in human cells with normal or low ascorbate concentrations did not require the involvement of p53 (*153, 208*). In addition to the promotion of chromosomal breaks via the activation of abnormal MMR, ascorbate was also a very potent enhancer of mutagenic activity of Cr(VI) (*141*). The ascorbate–Cr-DNA adduct is a unique form of DNA damage induced during Cr(VI) metabolism by vitamin C (*159*), and they were highly mutagenic during the replication of shuttle-vectors in human cells (*158*). MMR-promoted DSB were preferentially found in the G2 phase of the cell cycle, irrespective of doses, postexposure time, and type of cells (*141, 208*). G2 specificity of DSB production was caused by the requirement for Cr-damaged DNA to pass through the S-phase in order for the MMR system to activate aberrant processing (*141*). A combination of these findings led to the model that highly mutagenic adducts such as ascorbate–Cr-DNA cross-links induce mismatches during the replication of damaged DNA and that these compound lesions (mismatches at the site of Cr adducts) then lead to abnormal MMR (*141*). In this scenario, premutagenic adducts induce mutations and also promote larger chromosomal abnormalities (deletions and translocations) resulting from the error-prone repair of DSB through a nonhomologous end-joining process. Exposure of human cells to Cr(VI) is known to induce a series of gross chromosomal alterations, particularly in telomerase-negative primary cells (*220*).

The observed tolerance of Cr(VI) by MMR-deficient cells and the absence of MMR in chromate-induced lung cancers (*216, 219*) led to the formulation of the selection model of Cr(VI) carcinogenesis (*208, 221*) (Figure 6). This model postulates that chronic exposure to toxic doses of Cr(VI) results in the selective outgrowth of resistant clones that lack MMR. Once a population of these cells emerged, the subsequent exposure to Cr(VI) may no longer be necessary for the generation of additional mutations needed for the further progression of initiated cells because MMR-null cells have very high rates of spontaneous mutagenesis. Since p53 plays no significant role in the toxicity of Cr(VI) at biologically relevant doses (*153, 208*), there is no selective pressure to inactivate this tumor suppressor, and this could be the main reason why Cr-induced tumors retained wild-type p53. Overall, Cr(VI) carcinogenesis can be envisioned as a deadly combination of Cr-DNA damage processed by MMR into chromosomal abnormalities at low Cr(VI) doses and elimination of cells with an intact mutation avoidance mechanism at higher doses. Paradoxically, intracellular ascorbate is a very potent stimulator of both processes, leading to genomic instability in Cr(VI)-exposed cells (*141, 153*).

4.4. Cr(VI) as a Cocarcinogen. While there are well-documented situations of human exposure to Cr(VI) as a single agent, such as those encountered in chromate production, the majority of other occupational and probably all environmental exposures are actually coexposures with other carcinogens. Two examples of common coexposures are stainless steel welders and Cr(VI)-exposed workers who are also smokers. The possibility of cocarcinogenesis has been discussed for a long time, but the underlying considerations were largely theoretical, and only recently has chromate cocarcinogenesis been demonstrated in animal studies and the likely underlying mechanistic basis emerged. Two reports from Costa and colleagues (*222, 223*) provided strong experimental data demonstrating that Cr(VI) can act as a potent cocarcinogen for UV-induced skin tumors. In both studies, the presence of Cr(VI) in drinking water caused dose-dependent increases in the frequency of skin tumors in UV-irradiated hairless mice. Cr(VI) alone produced no tumors, indicating that it acted a strong enhancer of UV-initiated tumorigenesis. Supplementation with vitamin E or selenomethionine had no effect on Cr(VI)-mediated enhancement of skin carcinogenesis (*223*), suggesting that cocarcinogenic effects were not oxidant-mediated. The same regiment of antioxidants was very effective in suppressing As(III)-potentiated skin carcinogenesis in UV-irradiated hairless mice (*224*), demonstrating that Cr(VI) and As(III) enhanced UV-induced tumorigenesis via different mechanisms. While the evidence for Cr-UV cocarcinogenesis in mouse skin is very clear (*222, 223*), whether Cr(VI) reached skin cells through systemic distribution after the ingestion of Cr(VI)-laced water or whether skin was exposed to Cr(VI) externally remains uncertain.

The inability to repair UV-induced DNA damage leads to a dramatically increased risk of skin cancer and is the cause of xeroderma pigmentosum syndrome (*225*). Thus, one likely target of Cr-UV synergism could be interference of Cr(VI) with NER of pyrimidine dimers. Cr-DNA adducts are very good substrates for human NER (*184*). Thus, the presence of Cr-DNA adducts in UV-irradiated keratinocytes can be expected to divert NER machinery to the repair of relatively weakly mutagenic Cr-DNA phosphate modifications, which would increase the persistence of more mutagenic UV-DNA damage. It should be noted that exposures to even mildly or nontoxic Cr(VI) concentrations produce very high frequencies of Cr-DNA adducts (*141, 184*), which requires the prolonged involvement of many NER complexes to remove DNA-bound Cr complexes. For example, a 3-h treatment of primary human IMR90 cells with 2 μM Cr(VI), a dose corresponding to the current federal standard for Cr in drinking water, caused as much as 10^7 Cr-DNA adducts/cell (*184*). On the basis of the repair rate of $t_{1/2}$= 8.2 h in these cells, a full engagement of cellular NER would still leave about 10^6 Cr lesions/genome at 24 h postexposure (*184*). The reported comutagenicity of Cr(VI) and UV (*226*), which offers further support for the biological plausibility of Cr-UV

cocarcinogenesis, can be explained by the same mechanism involving the competition for NER factors.

Tobacco smoking and Cr(VI) exposure represent another potential case for synergistic tumorigenesis. Smoking is very common among chromate-exposed workers, and DNA adducts formed by polycyclic aromatic hydrocarbons, which are one of the main groups of tobacco-derived mutagens, are repaired by NER (*227*). Tang and co-workers (*228, 229*) have found pre-exposure to Cr(VI) indeed led to a significantly slower repair of BPDE-DNA adducts, which was accompanied by increased cytotoxicity and mutagenesis of BPDE. Coexposure of repair-deficient cells produced no synergism, thus establishing NER as the key target of enhancement of BPDE genotoxicity by Cr(VI). Interestingly, Cr(VI) appears to cause a selective increase in the number of BPDE adducts at the mutational hotspots of p53 in smoking-induced lung cancer: codons 248, 273, and 282 (*228*). These sites are likely to be repair coldspots, which would make them particularly sensitive to decreased NER because of the competition with Cr-DNA adducts. Although the majority of lung cancer cases among Cr(VI)-exposed workers were smokers (*135*), the interaction between smoking and Cr(VI) remained statistically uncertain because of the small number of cancer cases among nonsmokers. Despite good parallels between UV-Cr and BPDE-Cr comutagenesis, smoking-induced cancers have a more complex molecular etiology due to the presence of many carcinogens, some of which produce DNA damage that is not a substrate for NER (e.g., formaldehyde or nitrosamines, refs *230 and 231*). Cr(VI) and smoking-induced cancers also have different spectra of inactivated tumor-suppressors. While p53 mutations caused by activated polycyclic aromatic hydrocarbons and other mutagens are very common in tobacco-associated lung cancers (*232*), the majority of lung cancers in chromate workers retained wild-type p53 and instead inactivated the expression of MLH1 mismatch repair protein (*213, 216, 219*). These major molecular differences provide further support for epidemiological findings that despite its potential for cocarcinogenesis, Cr(VI) can act as a potent lung carcinogen by itself (*135*).

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