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Intracellular signal transduction of cells in response to carcinogenic metals

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Contents

Abbreviations: As^{3+}, arsenic(III); CDK, cyclin-dependent kinase; CDC25C, cell division cycle 25C; Cr^{6+}, chromium(VI); GADD45, growth arrest and DNA damage inducible protein 45; IKK, IκB kinase; JNK, c-Jun-N-terminal Kinase; NF-κB, nuclear factor-κB; ROS, reactive oxygen species; V^{5+}, vanadium(V).

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Abstract

Epidemiological and animal studies suggest that several metals and metal-containing compounds are potent mutagens and carcinogens. These metals include chromium, arsenic, vanadium, nickel, and others. During the last two decades, chemical and cellular studies have contributed enormously to our understanding of the mechanisms of metal-induced pathophysiological processes. Although each of these metals is unique in its mechanism of action, some common signaling molecules, such as reactive oxygen species (ROS), may be shared by many of the carcinogenic metals. New techniques are now available to reveal the mechanisms of carcinogenesis in precise molecular terms. In this review, we focused our attentions on carcinogenic metal-induced signal transduction pathways leading to the activation of NF-κB, cell apoptosis and cell cycle progression, three crucial steps or events involved in the transformation and carcinogenesis. This review summarizes current knowledge and our recent studies concerning intracellular signal transduction pathways initiated by carcinogenic metals and the cross-talk that occurs among these pathways in cells in response to metals.

Keywords: Metal; NF-κB; Carcinogenesis; Apoptosis; Cell cycle; Signal transduction

1. Introduction

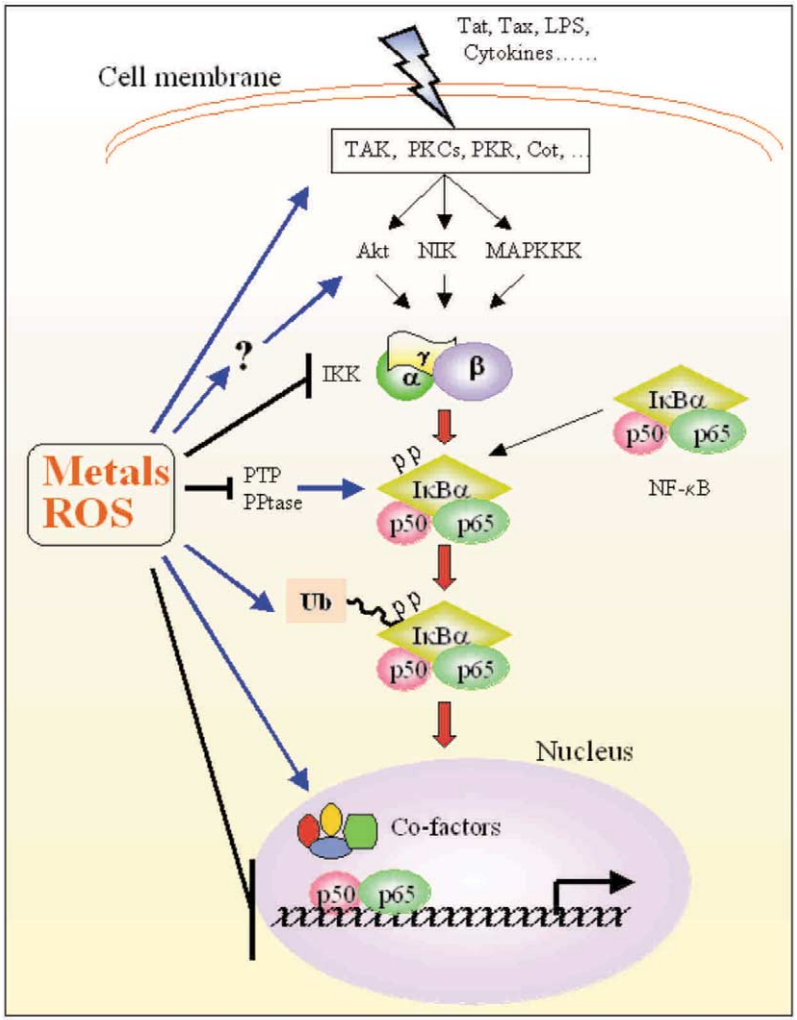
There is increasing evidence that many toxic metals or metal-containing particles from either environmental or occupational sources are human carcinogens [1–4]. Yet a detailed molecular mechanism of metal-induced malignant transformation and cancer remains elusive. Emerging evidence indicates that cellular transformation and tumorigenesis in humans is a multi-step process that requires both non-genetic and genetic alterations that promote the transformation of normal human cells into highly malignant tumors [5]. However, it is still not clear that which step or steps are targeted by the metals. For example, how the metals affect critical carcinogenic transformation events, such as NF-κB or apoptosis, is poorly understood. It is generally believed that oxidative stress, resulting from metal-induced generation of reactive oxygen species (ROS), is a critical mediator for the malignant transformation. However, ROS-independent effects of metals on cellular signaling pathway and genomic stability may also play a significant role [6,7].

It has been suggested that a cell undergoing malignant transformation requires at least three alterations: self-sufficiency in growth signals, escape from apoptosis and sustained cell cycling [5]. The question to be answered is how metals elicit these alterations. The carcinogenic effects of metals may be induced by targeting a number of cellular regulatory proteins or signaling proteins involved in cell growth, apoptosis, cell cycle regulation, DNA repair, and differentiation. This review will focus on pathways leading to the activation of transcription factor NF-κB, cell apoptotis and cell cycle regulation, three independent but interconnected pathways critically involved in carcinogenesis induced by metals, especially chromium, vanadium, arsenic, cobalt, copper, and nickel.

2. NF-κB activation by metals

2.1. General signaling pathway of NF-κB activation

A wide range of stimuli, which typically include cytokines, mitogens, various metals or non-metal particles present in the environment or work sites, intracellular stresses, viral and bacterial products, and UV light, induce expression of early response genes through the NF-κB family of transcription factors [8,9]. In resting cells, NF-κB is retained in the cytoplasm in its inactive form by interaction with one of a number of inhibitory molecules, including IκBα, IκBβ, IκBε, p105 and p100. Activation of the NF-κB signaling cascade results in complete degradation of IκB or partial degradation of the carboxyl termini of p105 and p100 precursors, allowing nuclear translocation of the NF-κB complexes. Activated NF-κB binds to specific DNA sequences in target genes, designated as κB-elements, and regulates transcription of genes mediating inflammation, carcinogenesis and anti-apoptotic reactions (Fig. 1).



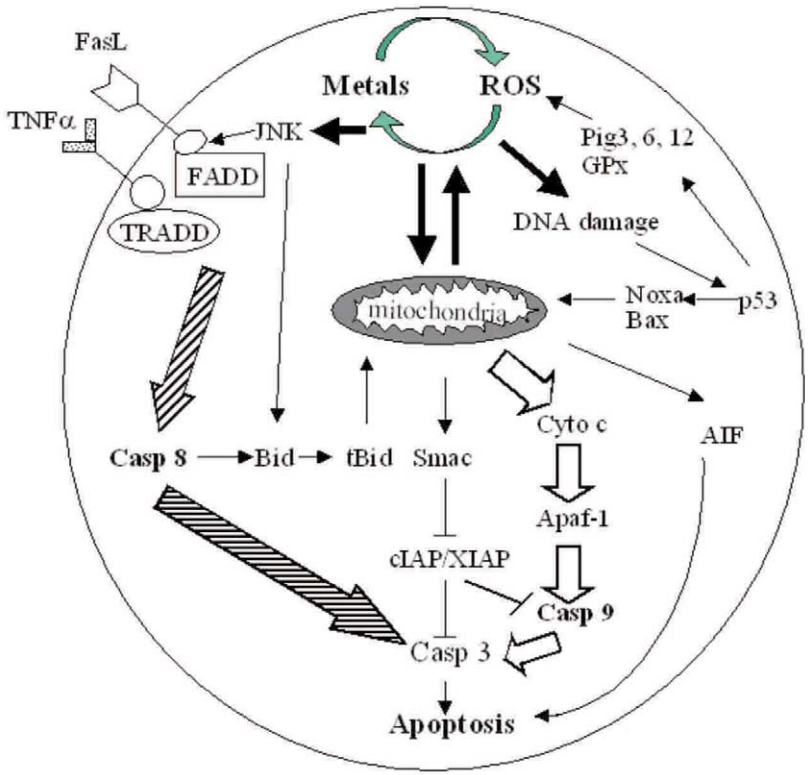


Fig. 1. Conflicting effects of metals or their ROS derivates on NF-κB signaling. Metals or ROS can possibly activate or potentiate the activation of up-stream kinases leading to the activation of IKK kinase complex. Meanwhile, metals or ROS can also inhibit IKK kinase activity through a direct modification of cysteine residues in the activation loop of IKK kinases. The phosphorylation of IκBα may be potentiated by metals or ROS due to the direct inhibition of tyrosine phosphatase (PTP) or serine/threonine phosphatases (PPtase) by metals or ROS. In the nucleus, metals or ROS may activate co-factors required for NF-κB-mediated transcriptional regulation on target genes, whereas they may also inhibit DNA binding activity of NF-κB by direct oxidation of critical cysteine residues in the DNA binding domain of NF-κB p50 or p65 proteins. →, activation; ⊣, inhibition.

Fig. 2. Signaling pathways of cell apoptosis induced by metals. Metals or ROS generated by metals directly initiate the intrinsic apoptotic pathway at the mitochondria (unfilled block arrows). Metals or ROS can also indirectly initiate this pathway by activating p53 as a result of metal- or ROS-induced DNA damage. The activation of p53 triggers the expression of Bax and Noxa, two apoptotic proteins that induce an increase in mitochondrial permeability. Cytochrome *C* is released into the cytosol from damaged mitochondria and binds to Apaf1, resulting in the activation of caspase-9. Damaged mitochondria can also release AIF, which induces apoptosis in a caspase-independent manner, and Smac which antagonizes the antiapoptotic effect of IAP proteins. Metals or ROS may also potentiate the extrinsic apoptotic pathway (shadow block arrows) through MAP kinase-, especially, JNK-mediated induction of FasL or Fas expression. Recruitment of death domain containing proteins, such as FADD and TRADD to these receptor complexes, activates caspase-8. The activation of caspase-8 and caspase-9 leads to the activation of the effector caspases, including caspase-3 and caspase-7.

IκBα is the most abundant inhibitory protein for NF-κB [9]. Signal-induced IκBα degradation involves phosphorylation of two serine residues, S32 and S36. This phosphorylation leads to polyubiquitination of two specific lysines on IκBα (K21 and K22) by SCF-β-TrCP complex and its degradation by the 26S proteasome (Fig. 1) [10]. The phosphorylation is accomplished by a specific IκB kinase (IKK) complex containing two catalytic subunits, IKKα and IKKβ, and a structural component named NEMO/IKKγ/IKKAP [11,12]. IKKα and IKKβ share 50% sequence similarity. Both proteins contain an amino terminal kinase domain, a carboxyl terminal region with a leucine zipper, and a helix-loop-helix domain. While In vitro and in vivo studies indicate that both IKKα and IKKβ are capable of phosphorylating IκBα on ser32 and ser36, IKKβ is more potent in IκBα phosphorylation induced by proinflammatory stimuli. Recent studies by several groups indicate the existence of an additional IKK-like kinase complex in T cells, named IKKi/ε [13,14]. IKKi/ε shares 27% homology with IKKα and IKKβ and possibly mediates NF-κB activating kinase (NAK) signaling and PMA/PKCε-induced S36 phosphorylation of IκBα and NF-κB activation.

2.2. Effects of metals on NF-κB activation

A number of reports during the last few years indicate that some metals are able to affect the activation or activity of NF-κB transcription factors [15]. To date, the results have been contradictory. Both activation and inhibition of NF-κB by metals have been reported [16–19]. Several studies from different groups indicate that, at a non-cytotoxic dosage, chromium (VI) (Cr^{6+}), arsenic trioxide (As^{3+}) or vanadium(V) (V^{5+}) are capable of activating NF-κB as monitored by either gel shift assay, reflecting the activation and nuclear translocation of NF-κB, or NF-κB-dependent reporter gene assay, an indicator of NF-κB activity. In contrast, it has been reported that Cr^{6+}, As^{3+}, and other metals inhibited NF-κB activation through interfering with IKK kinase, DNA binding, or the interactions with nuclear co-factor, cAMP-responsive element-binding protein (CREB)-binding protein (CBP) [18,19]. This controversy may largely result from the use of different doses of metals in each experimental system, since it is generally believed that high concentrations of metals are inhibitory for the activation and function of NF-κB.

2.3. Mechanisms of metal-induced NF-κB activation

The mechanistic basis of metal-induced NF-κB activation or its activity is also an unsolved issue, especially for Cr^{6+} and As^{3+}. In the case of V^{5+}-induced NF-κB activation, early study by Imbert and co-workers [20] indicated that the activation of NF-κB by V^{5+} occurred independently of IκBα degradation. However, this observation could not be reproduced by several recent studies, suggesting that V^{5+} did induce degradation of IκBα following the phosphorylation of serine or tyrosine [21,22]. In mouse macrophage cell line RAW264.7 cells, V^{5+} induced IκBα degradation occurred within 10–20 min with peak degradation at 40 min [21]. In human myeloid U937 cells or epithelial cells, V^{5+}-induced IκBα degradation occurred at 30 min and reached maximum at 240 min [22]. A similar result was achieved in Jurkat E6.1 cells and human B cell lymphoma line Ramos [23,24]. In contrast to earlier report that no resynthesis of IκBα occurred after V^{5+} treatment [20], several studies have indicated that the resynthesis of IκBα indeed occurred at 80–180 min after V^{5+} treatment [21,22].

Phosphorylation on either tyrosine 42 or serine 32/36 sites of IκBα has been demonstrated in cells treated with V^{5+} [21,22]. The phosphorylation of IκBα on these sites may contribute to the subsequent degradation of this protein. At present, there are no available data regarding the induction of IκBα phosphorylation and degradation in cells in response to other metals, such as Cr^{6+} and As^{3+}. The phosphorylation of tyrosine or serine residues on IκBα implies that certain tyrosine kinases and serine/theronine kinases are involved in metal-induced NF-κB activation. Since IKK is required for LPS- and inflammatory cytokine-induced IκBα phosphorylation and NF-κB activation, IKK may also contribute to metal-induced NF-κB activation. Indeed, in mouse macrophage cell line RAW264.7 cells, the activation of IKKβ kinase by V^{5+} is both dose- and time-dependent [21]. In comparison to cytokine- or LPS-induced IKKβ kinase activation, V^{5+} induces IKKβ activation in a relatively persistent manner.

It has been suggested that many metals, among which Cr^{6+} may be the most potent, can elicit an oxidative stress response in cells through the generation of ROS, including superoxide anion, hydroxyl radical, nitric oxide, and the by-product of superoxide, H_2O_2 [25–28]. High amounts of ROS generated from chronic and acute inflammatory responses or environmental stresses are cytotoxic. It is known that a limited production of ROS, as a consequence of electron transfer reaction in cytosol, peroxisomes and mitochondria, is buffered or scavenged by both enzymatic and non-enzymatic antioxidant systems inside cells [29]. The mechanisms of ROS generation by metals under biologically relevant conditions may involve Fenton/Haber–Weiss chemistry and autoxidation [28]. In macrophages or other phagocytes, metal-induced ROS generation may also involve the activation of hypochlorous acid, lipid peroxides and nucleoside hydroperoxides [30]. Studies using ROS scavengers or antioxidants have suggested

the involvement of cellular redox regulation on certain kinases, leading to the activation of NF-κB or other transcription factors [31]. In an early model, ROS-induced oxidative stress was proposed as a universal mechanism for NF-κB activation by diverse agents [32]. An unanswered and difficult question is which point in the activation pathway of NF-κB is targeted by ROS. The signal transduction pathways, such as the upstream and proximal kinases (e.g. IKK, for NF-κB activation induced by TNF, LPS or CD28) have recently been clearly identified [8,13]. However, no evidence has been presented to indicate that these kinase cascades would be oxidant-responsive or redox-regulated. Evidence provided by Li and Karin [33] demonstrated that the ROS scavenger, *N*-acetyl-L-cysteine (NAC), reduced TNFα-induced IκBα degradation and NF-κB DNA-binding activity, but failed to affect TNFα-induced IKK kinase activity in HeLa cells. These results raise the possibility that ROS may not target the activation pathway of IKK, but rather interfere with the steps of ubiquination and degradation of IκB. The model proposed by Roederer and co-workers [34] suggests that NF-κB activation was controlled by intracellular thiol levels in which NF-κB inducers somehow potentiate oxidative stress by depleting glutathione levels. However, this model contradicts several later observations, which showed that a glutathione-oxidizing agent could inhibit NF-κB through interference with its DNA binding [35–37]. Several studies indicate that cysteine 62 of the p50 subunit of NF-κB was essential for NF-κB DNA binding and that oxidation of cysteine 62 inhibited NF-κB DNA binding activity. Thioredoxin, a ubiquitous dithiol-reducing enzyme, can reduce cysteine 62 and restore DNA-binding activity of NF-κB [35]. It thus appears that the observed NF-κB activation by metal-induced ROS may depend on some alternative pathways, such as MAP kinases, Ras and Rac1, with the potential to stimulate the IKKs. Indeed, JNK inhibition by a transient transfection of macrophages with a dominant negative SEK1, an upstream kinase of JNK, decreased vanadate-induced IκBα degradation [21].

Many metals, including V^{5+} and As^{3+}, are potent inhibitors of protein tyrosine phosphatases (PTP) [38,39]. All PTPs contain a signature active site characterized with the sequence of His–Cys–X–X–Gly–X–X–Arg–Ser/Thr, where X is any amino acid [40]. Oxidation of the cysteine residue in this signature motif that is essential for the achievement of phosphatase activity will inactivate PTPs. Protein serine/threonine phosphatases, such as PP1, PP2A, PP2B and PP2C, may also be subject to redox regulation through the oxidative formation of a disulfide bond between a conserved pair of cysteine residues [41]. Since the extent of protein phosphorylation, such as IκBα phosphorylation, reflects the balance between the opposing actions of protein kinases and phosphatases, changes in either can consequently shift the extent of phosphorylation. It has been suggested that hypoxia and reoxygenation induces NF-κB activation through the induction of phosphorylation of tyrosine at position 42 on IκBα. Although this notion remains controversial, it will be important to determine whether tyrosine kinases are required for metal-induced NF-κB activation.

3. Metals induced cell apoptosis

3.1. General signaling pathways of apoptosis

Programmed cell death or apoptosis is a process in which cell death is initiated and completed in an orderly fashion through activation of various apoptotic pathways [42,43]. During the last decade, there has been an overwhelming interest in apoptosis and elucidation of mechanisms controlling this process. Apoptosis is an essential process required for development, morphogenesis, immune regulation, tissue remodeling, and some pathological reaction. Most apoptotic cells demonstrate characteristic morphological features, such as membrane blebbing, cell shrinking, cytosolic and nuclear condensation, and breakdown of chromosomal DNA. Depending on the use of different initiating caspases, signal-induced apoptosis can be roughly divided into two categories: receptor-mediated and mitochondrial-mediated apoptosis [42,43]. Whereas procaspase 8 is activated by receptors for Fas ligand and TNF through the recruitment of intracellular death domain-containing proteins, procaspase 9 is initiated by cytochrome *C* released from damaged mitochondria (Fig. 2). Both activated caspase 8 and caspase 9 use the same executive caspases, mainly caspase 3, to complete an apoptotic process. All caspases are expressed as inactive precursors and are activated by cleavage at specific peptide bonds. There are a number of cellular proteins which act as roadblocks in the activation cascade of caspases. These proteins act either by counteracting the effect on caspases, such as in the case of cIAP1, cIAP2 and XIAP, or stabilizing the outer membrane of mitochondria, such as in the case of Bcl-x and Bcl-2 [44,45]. The apoptosis mediated by mitochondria may be more important and relevant in metal-induced cell death.

Under normal conditions, mitochondria may be the main source for the generation of basic and homeostatic ROS through the chain reaction of electron transport. Paradoxically, mitochondria are also very vulnerable to the adverse effects of excessive generation of ROS. Excessive ROS that could not be neutralized by the limited sources of reducing capacity of the mitochondria and cytosol not only increase mitochondrial membrane permeabilization, but also diffuse into the micochondrial matrix to damage the respiratory

chain and consequently amplify the generation of ROS further due to the less efficient transferring of electrons to oxygen (O_2) [46]. Mitochondria with increased membrane permeability release several apoptosis-promoting factors including cytochrome *C*, apoptosis inducing factor (AIF), and Diablo/Smac [47–50]. Cytochrome *C* forms a complex with a cytosolic protein named apoptosis protease-activating factor-1 (Apaf-1) to activate caspase-9. AIF is a flavoprotein with homology to plant ascorbate reductases and bacterial NADH oxidases [50]. After release from the intermembrane space of damaged mitochondria, AIF exhibits a caspase-independent apoptotic function. Recently, another mitochondial intermembrane protein, Smac/Diablo, has been identified [48,49]. Once released from mitochondria to cytoplasm, Smac/Diablo binds to and antagonize cIAP1 and cIAP2, allowing the activation of caspases.

3.2. Metals and cell apoptosis

3.2.1. Cr^{6+}

Since apoptosis is an important factor influencing the malignant transformation of cells, the regulation of cell apoptosis may be critical in metal-induced carcinogenesis. The earliest report revealing apoptotic induction of Cr^{6+} was provided by Blankenship and co-workers [51,52] who demonstrated that cells treated with Cr^{6+} exhibited apoptotic features. Evidence provided by Singh et al. [53] indicated that depending on the rate, magnitude and spectrum of genotoxicity and mitochondrial damage, cells exposed to Cr^{6+} are fated to undergo either terminal growth arrest or p53-dependent apoptosis. It has been suggested that Cr^{6+} was able to damage DNA by forming DNA–Cr–DNA cross-links [6]. Cr^{6+} itself was found to be unable to react with macromolecules, such as DNA, RNA, proteins and lipids. Instead, Cr^{5+} or Cr^{3+}, intermediates of Cr^{6+} reduction, can form covalent interactions with DNA and other macromolecules [53], a process that activates DNA-dependent protein kinases (DNA-PK) and induces subsequent p53 activation and cell apoptosis. The DNA damaging effect of Cr^{6+} might be also through ROS generated during Cr^{6+} reduction. In support of this notion, recent studies from our laboratory have provided evidence showing that antioxidants, including pyrroline dithiocarbamate (PDTC) and aspirin, protected DNA from damage and p53 from activation induced by Cr^{6+} [54,55].

Activation of p53 tumor suppressor protein is considered to be one of the critical steps in the apoptosis induced by Cr^{6+} [51,55,56]. Several mechanisms are involved in Cr^{6+}-induced p53 activation. First, direct DNA damage by Cr^{6+} or ROS generated during cellular Cr^{6+} reduction activates upstream kinases, including DNA-PK, ATM, ATR and others, for p53 phosphorylation and activation [57,58]. Second, the p53 protein contains several redox-sensitive cysteines critical for the DNA binding activity of p53 [59,60]. As a transcription factor, p53 is able to up-regulate the expression of genes involved in either ROS production and metabolism, including quinone oxidoreductase (Pig3), proline oxidase (Pig6) homologues, glutathione transferase (Pig12), and glutathione peroxidase (GPx) [61]. Moreover, p53 also activates the expression of several genes that directly control or regulate the process of apoptosis. These genes include Bax, Fas, Fas ligand, IGF-BP3, PAG608 [62], ei24 (Pig8) [63], and Noxa [64]. The strongest evidence supporting the notion that Cr^{6+}-induced apoptosis depends on p53 was achieved by the use of fibroblasts from both wild type mice and p53-deficient mice [56]. Cr^{6+} induced two- to three-fold greater increase in apoptosis in wild type fibroblasts than in p53-deficient fibroblasts. Obviously, activation of p53 by Cr^{6+} will lead to the increased expression of p53-targeted apoptotic genes, such as Bax, Fas, Fas ligand, ei24, and Noxa. Several recent studies from both our group and others have highlighted the importance of H_2O_2 and hydroxyl radical in Cr^{6+}-induced p53 activation and cell death [28,30,55,65]. As discussed earlier, excessive ROS are toxic to mitochondria. Therefore, an alternative apoptotic pathway induced by Cr^{6+} may be through damage to mitochondria. Indeed, studies by Carlisle et al. [66] indicated that cyclosporin A, which prevents the pre-apoptotic release of cytochrome *C* from mitochondria, blocked Cr^{6+}-induced apoptosis and increased the survival rate of replication-competent but genetically damaged cells.

3.2.2. As^{3+}

Chronic exposure to As^{3+}, another important toxic metal (also named toxic sub-metal) widely found both in environment and in occupational settings, can cause a number of human diseases, especially, cancer [67,68]. The apoptotic effect of As^{3+} was originally observed in human acute promyelocytic leukemia cells [69]. Later studies suggested that As^{3+} might be a direct toxic agent for mitochondria in this type of cells. As^{3+} (1 μM) induced condensation of the mitochondrial matrix and disruption of the mitochondrial transmembrane potential leading to the release of cytochrome *C* and AIF [70,71]. As^{3+} can also induce apoptosis in other types of cells. Although the mechanism remains unclear, it has been consistently shown that p53 activation may not be involved in As^{3+}-induced cell apoptosis [69,72]. Based on studies using several antioxidants or ROS scavengers, two reports suggested an oxidative stress model for As^{3+}-induced apoptosis in Chinese hamster ovary cells [73,74]. In mouse skin epidermal cells, the apoptosis induced by arsenic is possibly through protein kinase C and MAP kinases pathways

[75]. MAP kinases, especially c-Jun-N-terminal kinase (JNK) and p38, have been proposed as important mediators in signal-induced apoptosis [75]. Both JNK- and p38-mediated cell death may be due to the induction of the rapid synthesis of FasL as observed in Jurkat cells [76], microglial cells [77] and hepatoma cells [78]. In addition, it has been demonstrated that JNK is also required for UV-induced caspase-independent proteolytic activation of Bid, a pro-apoptotic BH3-only member of the Bcl2 group that translocates to mitochondria after activation and induces cytochrome *C* release [79,80]. Our recent studies have found that JNK activation was required for arsenic-induced gene expression of GADD45, a cell cycle checkpoint protein arresting cells in G2/M phase transition, and the possible induction of apoptosis.

3.2.3. V^{5+}

Emerging evidence suggests that various forms of vanadium or vanadium containing particles from environmental and occupational sources are able to trigger or potentate cell apoptosis. It is believed that among various oxidation states, V^{5+}, one of the most common forms of vanadium found in nature and tissues or cells after exposure, is the most toxic [81]. Short-term exposure to V^{5+} causes local irritation of eyes and upper respiratory tract rather than systemic toxicity. There is limited information concerning the possible neoplastic transforming activity of V^{5+} following long-term exposure [81–83]. V^{5+} might cause apoptosis in some types of cells, whereas V^{5+} may be anti-apoptotic in other types of cells. In lymphoid cell lines, V^{5+} treatment caused the activation of caspases 3, 8 and 9, the induction of mitochondrial permeability transition, the release of cytochrome *C* and DNA fragmentation. Further studies suggest that there was no involvement of protein tyrosine kinase $p56^{lck}$ or phosphatase CD45 in this apoptotic effect of V^{5+} in lymphoid cell lines [84]. V^{5+}-induced apoptosis in mouse epidermal JB6 cells was thought to be involved in the generation of ROS and the activation of p53. Pre-treatment of the cells with antioxidants, including NAC and catalase, blunted the apoptotic effects of V^{5+} on these cells [85]. In other types of cells, V^{5+} might elicit an anti-apoptotic effect through the involvement of Akt/PKB activation or tyrosine phosphatase inactivation. Support for this comes from the work of Chin et al. [86] who showed that V^{5+} protected malignant glioma cells from apoptosis. Addition of wortmannin, a PI3 kinase inhibitor, abolished the protective effect of V^{5+} on these cells, indicating that PI3K and Atk/PKB may be responsible for mediating V^{5+}'s protective effect. In intestinal epithelial cells, studies by Scheving and co-workers [87] demonstrated a correlation between a decline in protein phosphatase activity and inhibition of cell apoptosis in V^{5+}-treated cells. It is not clear why V^{5+} is anti-apoptotic in some types of cells but is pro-apoptotic in other types of cells. The explanation for this may be the use of different forms of V^{5+}. It was noted that some studies used sodium vanadate whereas others used peroxovanadate or pervanadate. The later form is a reactive product of V^{5+} in the presence of H_2O_2. Several reports indicated that sodium vanadate and peroxovanade exhibit different effects on the induction of cell apoptosis, inactivation of protein phosphatase and generation of ROS [85,87]. An additional explanation for the contradictory effects of V^{5+} on cell apoptosis may be the use of different dosage of V^{5+}. It has been frequently observed that lower concentrations of V^{5+} are protective for cells from signal-induced apoptosis whereas higher concentrations of V^{5+} are either cytotoxic or pro-apoptotic. The final explanation for varying effects of V^{5+} on cell apoptosis is the types of cells used in each experiment. A well-known phenomenon is that cells originated from different tissues exhibit different capacities for the generation of ROS and respond differently to metal or exogenous ROS stimulation.

3.3. Relationship between apoptosis and carcinogenesis induced by metals

Evidence indicates that many of toxic metals, such as Cr^{6+}, As^{3+} and V^{5+}, are carcinogenic. It is difficult to reconcile the pro-apoptotic effects of metals with the observed carcinogenic potential of metals in humans. Many studies addressed the pro-apoptotic effect of metals on selected cell types, but failed to delineate how this process would contribute to cell transformation and carcinogenesis. Cell apoptosis was originally viewed as a normal process by which correct functional cellular population dynamics are maintained through the apoptotic loss of cell populations carrying abnormal genetic information [45]. It is known that metals under certain circumstances are apoptotic, but it is not known whether this apoptotic process induced by metals is a perfect or an imperfect process. An imperfect apoptotic process might result in the escape of cells that would be potentially carcinogenic. Thus, increased apoptosis under the conditions of chronic metal exposure would possibly increase the number of cells carrying damaged but replication-competent genetic information. Conversely, chronic and lower dose exposure of cells or tissues to metals may perturb or even inhibit appropriate apoptosis, leading to the accumulation of cells with carcinogenic potential. Among metals discussed above, it is likely that Cr^{6+} is not only the most toxic metal but also the most carcinogenic metal toward cells [6,15]. The genotoxicity of Cr^{6+} resulting from either direct DNA binding of Cr^{6+} derivatives or generation of ROS during cellular Cr^{6+} reduction may create a cell sub-population that may be predisposed to

mutagenesis and capable of averting apoptosis. Considering the fact that Cr^{6+}-induced apoptosis is p53 dependent under many circumstances, the mutations in the p53 gene will undoubtedly facilitate the development of apoptosis resistant and potentially carcinogenic cells. Moreover, although either Cr^{6+} itself or ROS generated from Cr^{6+} reduction intracellularly activates upstream signals leading to the accumulation and activation of p53 in the early phase of cells in response to Cr^{6+}, neither Cr^{6+} nor ROS can persistently activate the function of p53 protein in the later phase. Instead, due to oxidation of cysteine residues in the DNA binding domain of p53 protein [59,60], Cr^{6+} may actually inactivate the tumor suppressing function of p53 by impairing the DNA binding of p53. Support for this hypothesis has not yet been documented, but it should be plausible to explain why Cr^{6+} is able to activate p53 but is carcinogenic also.

4. Metals and cell cycle regulation

4.1. Cell cycle machinery

It has been known for decades that multiple signals exist to maintain proper cell growth and tissue homeostasis [88]. Most of the cells within a normal tissue may be forced out of the active cell cycling into the quiescent (G0) state from which they may reenter cell cycling under some future circumstances. In mature tissues, cells may undergo terminal differentiation by relinquishing their proliferative or cell cycling potential. In a variety of eukaryotic cells, the orderly progression of dividing cells through the G1, S, G2, and M phase of the cell cycle is controlled by a series of cell cycle regulatory proteins, mainly cyclins which exert their function by binding to and activating a number of specific cyclin-dependent kinases (CDKs). The CDK activity was further modulated by kinases and phosphatases that phosphorylate and dephosphorylate CDK, respectively. Moreover, several specific CDK inhibitory proteins and cell cycle checkpoint proteins, including $p21^{Waf1}$, $p16^{INK4a}$, $p27^{Kip1}$ [89] and GADD45, have been identified [90].

Emerging evidence during recent years has demonstrated that a variety of stress inducers, including DNA-damaging agents, activate checkpoint function of cells, leading to a cell cycle arrest. There are several checkpoints, existing in G1/S phase, G2 phase and M phase of cell cycle (Fig. 3). These checkpoints have surveillance systems to detect specific DNA structures indicative of damage or ongoing repair and replication. An intracellular signal transduction cascade is then initiated that either blocks S phase by inhibiting the activity of cyclin D/CDKs that phosphorylates retinoblastoma protein (RB) or that blocks the onset of mitosis by maintaining Cdc2 tyrosine phosphorylation and preventing CDK activation. In mammalian cells, the control of S phase checkpoint requires the p53 tumor suppressor protein that governs the expression of CDK inhibitors, p21 [57,91]. The activation of G2/M phase checkpoint is dependent on the phosphorylation and inactivation of CDC25C phosphatase by checkpoint kinases 1 or 2 (Chk1 or Chk2) and the induction of GADD45, an inhibitor for G2/M phase cyclin B/CDC2 complex [92,93]. An additional checkpoint, the spindle checkpoint, has been identified in a later stage of M phase [88,89]. This checkpoint arrests mitotic progression if the spindle is not properly assembled, or if the chromosomes are not correctly oriented and attached to the spindle. All of the checkpoints are essential for maintaining genomic stability by allowing cells to have enough time to repair damage and thus protect the organism from the deleterious consequences of mutation.

4.2. Metals and cell cycle

Increasing evidence suggests that the components of the cell cycle machinery are frequently altered during carcinogenic transformation of cells [94]. Given the fact that many toxic metals are carcinogens or suspected carcinogens, it may be that the carcinogenic potential of metals may be achieved by affecting the expression and function of cell cycle regulatory proteins, especially cell cycle checkpoint proteins that maintain genomic

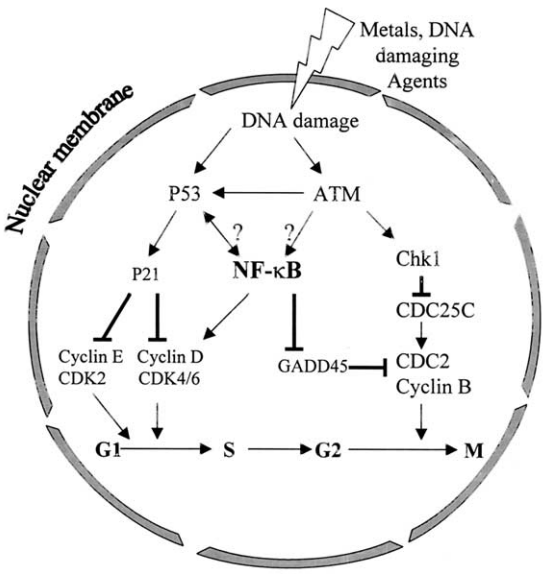


Fig. 3. Cell cycle regulation by metals. Metals or other DNA damaging agents may activate p53 and ATM through the activation of DNA damaging-dependent protein kinase. Cell cycle arrest may occur at G1 to S phase transition due to the p53-dependent induction of p21, an inhibitor for both cyclin E/CDK2 and cyclin D/CDK4/6 complexes. G2/M phase arrest may be induced also by metals, because of Chk1 activation and subsequent inhibition of CDC25C.

stability and integrity of cells in response to environmental stresses, both genetically and epigenetically. Unfortunately, much of our understanding of the effects of metals on the cell cycle comes from a few scattered studies and speculations. It is known that some metals or their ROS derivatives are capable of damaging DNA [19,65]. In addition, some toxic metals, such as V^{5+} and As^{3+}, are protein phosphatase inhibitors [38,87]. Dual specific protein phosphatases, including CDC25A, CDC25B and CDC25C, are required for progression from G1 to S phase and G2 to M phase, respectively. Inhibition of the phosphatase activity of CDC25 family members by metals will delay the cell cycle transition. Several lines of indirect evidence support this hypothesis. V^{5+} treatment of live cells transfected with a CDC25B-green fluorescent protein construct arrested cells in G2/M phase and inhibited nuclear translocation of CDC25B [95]. Possible G2/M cell cycle arrest induced by As^{3+} has been observed in myeloma cells [96], fibroblast [97], and lymphocytes [98]. Our recent studies provide the strongest evidence for a G2/M-arresting effect of As^{3+} in human bronchial epithelial cell line BEAS-2B cells transfected with a wild type or kinase-mutated IKK expression vector (unpublished observations). First, flow cytometric analysis demonstrated that at 48 h following As^{3+} treatment, BEAS-2B cells showed a marked dose-dependent increase of cells arrested in G2/M phase and a corresponding decrease in the number of cells in G1 phase. This effect of As^{3+} was further potentiated by NF-κB inhibition. Second, a dose-dependent induction of GADD45 protein was observed in cells treated with arsenic. This induction of GADD45 by arsenic appears to be depended on the activation of JNK, since blockage of JNK activation by expression of a dominant negative SEK1 vector decreased the induction of GADD45. Third, analysis for the expression of CDC25 family members revealed that arsenic induced CDC25A expression but markedly reduced the levels of CDC25B and CDC25C proteins, two phosphatases dephosphorylating and activating the CDC2/cyclin B complex required for the transition of the cell cycle from G2 to M phase. The effects of Cr^{6+} on the regulation of cell cycle were also determined and appear to be more complicated. Similar to As^{3+}, Cr^{6+} was able to induce GADD45 and suppress CDC25B and CDC25C. In contrast to As^{3+}, Cr^{6+} had no effect on CDC25A. Cell cycle profiling studies showed that, whereas a lower concentration of Cr^{6+} (0.25 μg/ml) promoted cell cycle transition, higher concentrations of Cr^{6+} (1–4 μg/ml) arrested cells at S phase. In the case of V^{5+}-induced cell cycle regulation, the cell cycle arresting effect of V^{5+} seems to be dependent on the status of NF-κB activation. In normal epithelial cells, V^{5+} exhibited less effect on cell cycle transition. However, in the cells where NF-κB activation was specifically inhibited, V^{5+} showed a remarkable G2/M phase arresting effect. Nevertheless, V^{5+} was unable to induce the expression of GADD45, an inhibitor of cyclin B/CDC2 complex required for G2/M transition.

4.3. Cell cycles and tumorigenesis

The activation of cell cycle checkpoints leading to cell cycle arrest reflected reactions of cells in response to environmental stresses. A defect in checkpoint function will undoubtedly increase the chance of tumorigenesis. However, activation of checkpoints by either toxic metals or their ROS derivatives does not necessarily mean that the potential for mutation or carcinogenic transformation will be overridden. Depending on the duration and severity of damage, the repair process may either be completed or not after the activation of checkpoint signals. Since metals are non-biodegradable and persist after uptake by cells or tissues, the cells will be unable to repair damage created by the exposure of metals due to the exhaustion of their checkpoint and repairing systems. The consequence will be either an unavoidable cell death or tumorigenesis.

5. Cross-talk among signaling pathways in cells exposed to metals

We discussed three different signaling pathways leading to the activation of NF-κB, apoptosis and cell cycle regulation that are potentially involved in metal-induced carcinogenic transformation. Each of these pathways has unique signaling molecules to sense upstream signals and relay these signals to downstream effectors. It is now clear that all of these signaling pathways are neither isolated nor transduced in a linear fashion from one molecule to the next as previously expected. Cross-talk among different signaling pathways is becoming increasingly important for our understanding of signaling networks. Cross-talk can take place at many levels from the membrane to the cytosol, to the nucleus and involve components that are common among several pathways, as well as positive and negative feedback signals that can act at many steps among different pathways from kinases to transcription factors (Fig. 4).

5.1. NF-κB and apoptosis

Several metals, such as As^{3+} and V^{5+}, can induce the activation of NF-κB and cell apoptosis simultaneously under many circumstances. The question to be answered is that is NF-κB a mediator of metal-induced apoptosis or an innocent bystander or a protector of cells from metal-induced cell death? To answer this question, the first thing needed to be understood is what is the general relationship between NF-κB and apoptosis.

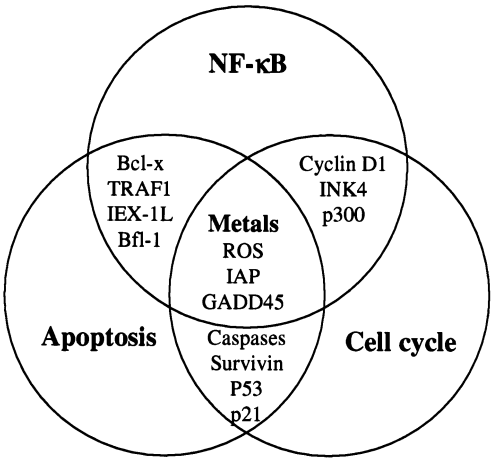


Fig. 4. Cross-talk among signals of NF-κB, apoptosis and cell cycle.

5.1.1. Interaction of NF-κB and p53

There are already well-established links between NF-κB activation and cell apoptosis. A reciprocal relationship between NF-κB and p53, a major player in apoptosis, had been noted in several recent studies. NF-κB could directly counteract the function of p53 through either competing with coactivator CREB-binding protein or sequestering p53-binding protein 2 [99,100]. Studies conducted by Jung and his colleagues [101] indicate that ATM was involved in IκBα phosphorylation in SV40 large T-transformed fibroblasts in response to ionizing radiation. In non-transformed fibroblasts, however, Ashburner et al. [102] demonstrated a lack of involvement of ATM in IκBα phosphorylation. In an in vitro study, Liu et al. [103] reported that DNA dependent protein kinase (DNA-PK), a kinase phosphorylating p53 in response to DNA damage, is able to phosphorylate the carboxyl terminus of IκBα protein. In our recent studies, we found that Cr^{6+} induced an elevation of N-terminal phosphorylation of the p53 protein in IKKβ-inhibited cells accompanied by a remarkable increase of cell death. Amino acid sequence search identified a similarity of kinase domain between IKKβ and Chk2, a kinase capable of phosphorylating Ser^{20} of p53 protein [104,105]. These raises a possibility that both IKKβ and Chk2 may share a common up-stream kinase for their activation or select the same substrate in response to oxidative or other stress signals.

5.1.2. NF-κB is an anti-apoptotic transcription factor

Based largely on earlier studies, NF-κB was initially considered as a pro-apoptotic factor due to its rapid activation in cells in response to apoptotic signals and its involvement in the expression of some apoptotic genes, including TNFα, c-myc and fasl [106,107]. More recent work, however, has altered this view and revealed an anti-apoptotic effect of NF-κB in response to a variety of apoptotic stimuli. RelA (p65) deficient mice die during embryonic development through apoptosis of hepatocytes [108]. Disruption of IKK genes further supports an anti-apoptotic role for NF-κB. IKKβ knockout mice die as embryos and show massive liver cell apoptosis [109,110], a phenotype similar to the response of NF-κB p65 gene knockout mice; whereas knockout of the IKKα gene resulted in perinatal lethality of mice with an increased the thickness of the skin due to the deficiency of keratinocyte differentiation [111]. Male mice with an inactivated X-linked gene encoding IKKγ/NEMO, an essential modulator of the IKK complex for NF-κB activation, died at mid-gestation due to a massive cortical and medullary lymphocyte apoptosis in the thymus in addition to degeneration of the liver [112,113]. Female mice deficient in the IKKγ/NEMO gene manifested a unique dermatopathy due to the apoptosis of keratinocytes and consequent abnormal pigmentation, a characteristic strikingly similar to that of the human X-linked dominant, male-lethal genetic disease—incontinential pigmenti or Bloch–Sulzberger Syndrome. Activation of NF-κB is known to be required for the induction of survival genes or anti-apoptotic genes whose products can block stress signal-induced cell death, a process critically involved in cell proliferation and transformation. Candidate anti-apoptotic genes targeted by NF-κB include those encoding the cell cycle regulatory protein cyclin D1[114–116], the mitochondrion membrane stabilizing proteins Blf-1 and Bcl-x [117,118], the caspase inhibitors cIAP1/cIAP2 and XIAP, and the TNF receptor-associated factors TRAF1 and TRAF2 [119]. It should be noted that several reports suggested that NF-κB was also a pro-apoptotic factor in FasL-induced cell death [120,121]. This argument is largely based on earlier observations that NF-κB can regulate the artificial promoter activity of the FasL gene, a gene encoding an important activator of apoptosis through a CD95/Fas- and Fas-associated death domain (FADD)-mediated caspase 8 activation pathway [43]. However, both promoter truncation studies of the FasL gene and somatic cell mutagenesis studies of IKKγ indicate that NF-κB is not required for the FasL gene expression [122,123].

5.1.3. NF-κB protects cells from metal-induced cell death

As discussed earlier, the protective roles of NF-κB from signal-induced cell death may be related to its transcriptional regulation on several anti-apoptotic genes, such as bcl-xl, Al, cIAP1, cIAP2, and some of TNFα receptor associated proteins [117,118]. To delineate the molecular events of NF-κB-mediated regulation of Cr^{6+}-induced cell death, we have recently generated cell lines by stable transfection of wild-type IKKβ and kinase mutated IKKβ (IKKβ-KM), respectively. In the cells stably expressing IKKβ-KM, an

essential component of NF-κB signaling, IKKβ, is defective (Chen et al., unpublished observation). Cell morphologic analysis indicates that treatment of the cells expressing IKKβ-KM with Cr^{6+} induced a necrotic-like cell death. Although IKKβ-KM expressing cells exhibited basal activation of caspase-3 and cleavage of Bcl-x protein, no appreciable apoptotic morphologic changes were observed under this basal condition. Similar phenomena were observed in mouse embryo fibroblasts from IKKβ gene deficient mice (Chen F, Li Z, Karin M, and Shi X, manuscript in preparation). Global gene expression profiling analysis shows that inhibition of IKKβ to block NF-κB signaling decreased the expression of two important anti-apoptotic genes, cIAP1 and cIAP2. In the cells transfected with a control vector or IKKβ, we observed a dose-dependent activation of NF-κB by Cr^{6+}. Thus, the activation of NF-κB may account for a protective effect on the cellular response to metals, such as Cr^{6+}.

5.2. NF-κB and cell cycle

5.2.1. Contributions of NF-κB to cell cycle

The relationship between NF-κB and apoptosis has been intensively explored during the last few years, whereas only limited information is available regarding the possible involvement of NF-κB in cell cycle regulation in response to a variety of stress signals. Several reports indicate that NF-κB activation was required for cells to re-enter G1 from G0 in mouse fibroblasts and in regenerating liver [124–127]. It was found that the levels of NF-κB activation were linked to signaling that controls cell cycle progression in HeLa cells and Jurkat T cells [128,129]. Inhibition of NF-κB caused impairment of cell cycle progression in human glioma cells [130] and a retarded G1/S transition in HeLa cells [131]. Direct evidence indicating the contributions of NF-κB to the cell cycle comes from studies of cyclin D1 gene expression [114–116,132]. Cyclin D1, in association with CDK4 and CDK6, promotes G1/S phase transition by phosphorylating the retinoblastoma protein (pRB), thereby releasing the transcription factor E2F, which is required for the activation of S phase specific genes [133–135]. Two NF-κB binding sites in the human cyclin D1 promoter have been identified that conferred activation by NF-κB as well as by growth factors. Inhibition of NF-κB by a degradation resistant IκBα caused a pronounced reduction of serum-induced cyclin D1 expression accompanied by a decrease of cyclin D1-associated kinase activity and delayed phosphorylation of the pRB. On the other hand, certain cell cycle regulatory proteins could also modulate the transcriptional activity of NF-κB through a coactivator, p300 [128]. It was found that an amino-terminal region of p300 interacted with the transactivation domain of p65 subunit of NF-κB complex, whereas a carboxyl-terminal region of p300 preferentially interacted with cyclin E-CDK complex. The CDK inhibitor, p21, or a dominant negative Cdk2 stimulated NF-κB-dependent gene expression. In resting cells, NF-κB was inactive, since it is bound to its inhibitor, IκBα, that contains several ankyrin repeat domains. Intriguingly, it was found that the inhibitor of Cdk4 (INK4) contains ankyrin repeats and exhibited IκB-like function by binding and inhibiting NF-κB [136].

5.2.2. Metal-induced NF-κB activation and cell cycle regulation

In agreement with above notions, our recent studies in human bronchial epithelial cell lines have found that NF-κB and JNK are reciprocal regulators of G2/M cell cycle arrest in cells treated with As^{3+} or Cr^{6+}. Interruption of the NF-κB signaling pathway by stable expression of a kinase mutated form of IKKβ potentiated the induction of GADD45 and G2/M cell cycle arrest induced by As^{3+} or Cr^{6+}. In contrast, interruption of the signal transduction pathway leading to the activation of JNK decreased the inducible expression of GADD45 in cells treated with As^{3+}. These results strongly suggest that in response to toxic metals, activation of NF-κB appears to be unfavorable for the induction of cell cycle arrest. Therefore, a persistent activation of NF-κB in cells in response to metals might facilitate the development of carcinogenic transformation.

5.3. Apoptosis and cell cycle

5.3.1. Linkages between the cell cycle and apoptosis

The signaling pathways for both cell cycle regulation and apoptosis appear distinct, but a great deal of cross-talk between these two processes has been discovered [137]. The cross-talk can occur at multiple levels. First, many extracellular signals for cell cycle regulation also affect cell apoptotic pathways. Second, both apoptosis and cell cycle regulation share a number of common intracellular signaling components. The best example involves the signals from DNA damaging agents. DNA damage activates p53 that either trigger cell cycle arrest in some types of cells or apoptosis in others. Following p53 activation, cell cycle progression was arrested by the induction of the CDK inhibitor p21 [91] or CDC25C sequester 14-3-3δ [138,139], and the apoptotic signaling cascade was initiated due to the expression of p53-dependent apoptotic genes. Apoptotic genes regulated by p53 include Bax, Noxa, and Pig3 [140], which are capable of activating the Apaf-1-caspase 9 apoptotic pathway through an increase of mitochondrial membrane permeability and subsequent release of cytochrome *C*, AIF and Smac/Diablo [48,50]. Another indication of the linkages between the cell cycle and apoptosis involves survivin, an anti-apoptotic

protein within IAP family. Compared with other IAP family members, survivin contains a single baculovirus IAP repeat and lacks a carboxyl-terminal RING finger domain. As an IAP family member, survivin is able to inhibit the activation of caspase 3 and caspase 7 through its IAP repeat domain in a cell free system. It has been observed that survivin was expressed preferentially during the G2/M phase and was associated with mitotic spindle microtubules during metaphase and with midbodies during late telophase [141–143]. Disruption of survivin expression by gene targeting resulted in a deficiency of cell proliferation, a strong indication that survivin participates in cell cycle regulation [144]. A recent report by Suzuki and co-workers [145] demonstrated that survivin initiated S phase progression by removing p16INK4a, a known specific suppressor for Cdk4 and Cdk6, from Cdk4.

Based on the observation showing that CDC2, CDK4 and/or CDK6 are upregulated in cells undergoing apoptosis, a number of studies suggest that CDK activity is not only essential for cell cycle progression but is important for promoting apoptosis also. Manipulations that decrease CDK activity prevent apoptosis, and those of which enhance CDK promote apoptosis [140,146,147]. It has been speculated that CDK might function as part of a positive-feedback loop that leads to phosphorylation of apoptosis-promoting proteins resulting in further activation of caspases. Evidence that caspase cleaves p21 and p27, inhibitors of CDKs, reversed this hypothesis. This study suggested that CDK activation during apoptosis was a consequence of caspase-mediated degradation of CDK inhibitors, rather than a cause of apoptosis [148–150]. Indeed, activated caspase-3 during apoptosis has been implicated in proteolysis of a number of cell cycle regulatory or machinery proteins, such as mitotic spindle proteins [151]. Therefore, expression of intracellular inhibitors of apoptosis, including cIAP1, cIAP2, XIAP and survivin, will indirectly regulate the processes of cell cycle.

5.3.2. Metals and cell cycle regulatory proteins

Studies by Yang and his co-workers [152] indicated that some IAP family members, in addition to their inhibitory activity on caspases to block apoptosis, could exhibit ubiquitin ligase activity and promote protein degradation. Many mammalian cell cycle machinery proteins and regulatory proteins, such as cyclins, p27, p21, E2F, CDC25A, Rb and p53, are known to be ubiquitinated by ubiquitin ligase and degraded by proteasome [153–155]. Although direct evidence is still missing, it is highly likely that inhibitors of apoptosis including cIAP1 and survivin may regulate cell cycling by affecting the stability of cell cycle proteins. Both cIAP/XIAP proteins and traditional ubiquitin ligases contain a metal-coordinating, redox sensitive C3H2C3 or C3HC4 RING finger domain (C, cysteine; H, histidine) in their carboxyl terminus [152]. Our recent studies indicate that in As^{3+} treated cells, the levels of ubiquitinated CDC25C is concomitantly increased with the levels of cIAP1 proteins (Chen et al., unpublished). We assume that this effect of arsenic on CDC25C ubiquitination and degradation is neither a coincidence nor simply a reflection of random protein turnover. Rather, it indicates an altered ubiquitin ligase activity in cells in response to metals or ROS derivatives. It will be important to determine whether NF-κB-dependent expression of IAP proteins contribute to the ubiquitination and degradation of CDC25C and consequent G2/M cell cycle arrest.

6. Summary

One of the major challenges in understanding mechanisms of carcinogenic transformation of cells in response to toxic metals is to elucidate how signal transduction pathways are activated and how signaling cross-talk and specificity are achieved when several signaling pathways that elicit different cellular responses are activated at the same time by metals. For instance, why does activation of the NF-κB, an anti-apoptotic transcription factor, coincide with obvious apoptotic features in cells treated with metals, whereas activation of NF-κB seems to be unfavorable for the induction of cell cycle arrest required for the maintenance of genomic stability. Since metals or their ROS derivatives are highly reactive but non-specific molecules, an activation of only one specific signaling pathway, for example NF-κB or apoptosis, is hard to achieve in the cells in response to metals. Even in a single signaling pathway, because of their highly reactive and non-specific characteristics, metals or ROS can in principle induce conflicting signals by interfering with signaling molecules at different levels. A good example is the NF-κB pathway. It has been frequently observed in certain type of cells that oxidative stress amplified or potentiated NF-κB activation, whereas at the same time oxidation of NF-κB proteins inhibited NF-κB function. Translating the knowledge gained by studying the connections among NF-κB activation, cell apoptosis and cell cycle regulation may aid in developing novel preventive measures and therapies for diseases related to environmental and occupational exposures to metals.

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