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Molecular Mechanisms of Chromium (VI) - Induced Toxicity and Carcinogenesis

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**Molecular Mechanisms of Chromium (VI) - Induced
Toxicity and Carcinogenesis**

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Dissertation submitted to the School of Pharmacy at West Virginia
University in partial fulfillment of the requirements for the degree of

**Doctor of Philosophy
in
Pharmaceutical and Pharmacological Sciences**

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Keywords: chromium (VI), cancer, lung, apoptosis, Bcl-2, malignant transformation, reactive oxygen species, interactome, Ingenuity

ABSTRACT

Molecular Mechanisms of Chromium (VI) - Induced Toxicity and Carcinogenesis

DJORDJE MEDAN

Cr(VI) compounds are redox cycling carcinogens that induce apoptosis as a primary mode of cell death. Since defects in apoptosis regulatory mechanisms are considered to be indispensable foundation of carcinogenesis it is critical to understand the molecular effectors involved. Cellular energy powerhouses, the mitochondria, house the effectors involved in signaling cascades comprising commitment to apoptosis and are reported to be dysregulated in variety of tumors. The objective of this study was to characterize the key signaling effectors in Cr(VI)-induced apoptosis and determine the relationship between those effectors and mode of cellular energy production in cancerous vs. non-cancerous cells. Our data show that Cr(VI) induced mitochondrial ROS dependent apoptosis in human lung cancer (H460) and non-cancerous (BEAS-2B) cells. The intensity of mitochondrial ROS production and consequently apoptosis was highly dependent on the energy substrate available to the cells. Forcing of oxidative phosphorylation-only energy metabolism abrogated apoptosis resistance in cancerous H460 cells vs. non-cancerous BEAS-2B cells. Collectively, these findings present a mechanistic advancement in the understanding of molecular effectors underlying the Warburg's "mitochondrial dysregulation" hypothesis and put mitochondrial ROS as key mediators causal agents of the commitment phase of programmed cell death in a model of Cr(VI)-induced toxicity. In addition, long-term exposure to Cr(VI) leads to malignant transformation of human lung epithelial BEAS-2B cells, as indicated by their increased cell migration, invasion, proliferation, and colony formation activities. Cr(VI)-transformed cells induced tumorigenesis in nude mice comparable to that of well established human lung cancer H460 cells, whereas passage-matched control BEAS-2B cells showed no

tumor development. To provide a mechanistic insight to the tumorigenic process, stable knockdown mutants of Cr(VI)-transformed cells and H460 cells exhibiting downregulated Bcl-2 phenotype were generated and evaluated for tumor associated properties in vitro and in vivo. The Bcl-2 knockdown mutants showed a substantial decrease in the rates of colony formation, invasion, migration and proliferation, as well as tumor formation in mice, in comparison to vector controls. These results indicate a novel role of Bcl-2, beyond apoptosis regulation, to be a key regulator of malignant transformation and tumorigenesis in vivo. The model described here may provide utility to other xeno-carcinogenesis studies, including those of heavy metals which currently lack effective in vivo tumor development experimental means.

Dedicated to

Peter D. Mitchell
(1920-1992)

Whose example of ingenuity, creativity, courage and perseverance is a true testament to the Giants of science on whose shoulders we stand today.

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TABLE OF CONTENTS

ABSTRACT.....	ii
DEDICATION	iv
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vi
LIST OF FIGURES	viii
ABBREVIATIONS	x
CHAPTER 1. Background and Objective	1
1.1 The Warburg Paradox	2
1.2 Chromium Exposure	3
1.3 Chromium Metabolism	4
1.4 Chromium (VI)-Induced Oxidative Stress	5
1.5 Chromium (VI)-Induced DNA Damage and Mutagenesis	7
1.6 Summary	12
1.7 Specific Aims	13
1.8 References	14
CHAPTER 2. Energy Substrate Modulates Cr(VI)-Induced Apoptosis Differentially in Cancerous vs. Non-Cancerous cells via a Mitochondrial- Reactive Oxygen Species Dependent Mechanism	20
2.1 Abstract	21
2.2 Introduction	22
2.3 Materials and Methods	24
2.4 Results	27
2.5 Discussion	31
2.6 References	47

CHAPTER 3. Malignant Transformation and Tumorigenesis of Chromium (VI)-Exposed Lung Cells and their Inhibition by Bcl-2 Knockdown	53
3.1 Abstract	54
3.2 Introduction	55
3.3 Materials and Methods	56
3.4 Results	60
3.5 Discussion	64
3.6 References	76
OVERALL CONCLUSIONS	79
CURRICULUM VITAE	80

LIST OF FIGURES

1.0 Background and Objectives

Figure 1.	Schematic Representation of Cellular Energy Metabolism	9
Figure 2.	Diagrammatic Illustration of Oxidative Phosphorylation Pathway.....	10
Figure 3.	Schematic representation of known cellular reduction and cytotoxic effects of Cr(VI).....	11

2.0 Energy Substrate Modulates Cr(VI)-Induced Apoptosis Differentially in Cancerous vs. Non-Cancerous cells via a Mitochondrial-Reactive Oxygen Species Dependant Mechanism

Figure 1.	Induction of apoptosis by Cr(VI) in human lung epithelial BEAS-2B and human lung cancer H460 cells	36
Figure 2.	Effect of energy substrate on Cr(VI)-induced apoptosis in human lung epithelial BEAS-2B and human lung cancer H460 cells	37
Figure 3.	Cr(VI)-induced ROS production and mitochondrial membrane depolarization.....	38
Figure 4.	Effect of energy substrate on ROS production and mitochondrial membrane depolarization	39
Figure 5.	Effect of ROS modulators on Cr(VI)-induced apoptosis in BEAS-2B and H460 cells	40
Figure 6.	Localization of ROS involved in Cr(VI)-Induced Apoptosis.....	41
Figure 7.	GPx and MnSOD overexpression increases cell death resistance to Cr(VI).....	42
Figure 8.	Bcl-2 overexpression decreases Cr(VI)-induced mROS and apoptosis.....	43
Figure 9.	Prevailing model of carcinogenesis.....	44
Figure 10.	Mitochondrial ROS production and defense	45

Figure 11. Proposed mechanism contributing to Cr(VI)-induced toxicity and carcinogenesis	46
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3.0 Malignant Transformation and Tumorigenesis of Chromium (VI)-Exposed Lung Cells and their Inhibition by Bcl-2 Knockdown

Figure 1. Cell growth and colony formation capacities of BEAS-2B, BEAS-Cr and H460 cells	68
Figure 2. In vitro relative malignant phenotype capacities of BEAS-2B, BEAS-Cr and H460 cells	69
Figure 3. Bcl-2 expression and response to Cr(VI) treatment in BEAS-2B, BEAS-Cr and H460 cells	70
Figure 4. Effect of Bcl-2 knockdown on tumor-associated properties <i>in vitro</i>	71
Figure 5. Effect of Bcl-2 knockdown on tumor-associated properties <i>in vivo</i>	72
Figure 6. Ingenuity Pathways Analysis software output for the Bcl-2-interactome.....	73

ABBREVIATIONS

Cr, chromium; Cr(III), trivalent chromium; Cr(VI), hexavalent chromium; IARC, International Agency of Research on Cancer; ATCC, American Type Culture Collection; Bcl-2, B-cell lymphoma-2; ROS, reactive oxygen species; mROS, mitochondrial ROS; $\cdot\text{O}_2^-$, superoxide anion; H_2O_2 , hydrogen peroxide; $\cdot\text{OH}$, hydroxyl radical; NAC, *N*-acetyl cysteine; DPI, diphenylene iodonium; MnSOD, manganese superoxide dismutase; GPx, glutathione peroxidase; BCA, bicinchoninic acid; BSO, buthionine sulfoximine

CHAPTER I

Background and Objectives

1.1 *The Warburg Paradox*

Ever since Otto Warburg's 1930 observation that cancer cells prefer to derive energy from anaerobic glycolysis as opposed to, energetically speaking, much more efficient oxidative phosphorylation (Figs. 1-2), elucidation and confirmation of molecular mechanisms underlying this apparent anomaly have been underway. Although the glycolytic phenotype of many cancer cell lines and tumors has been demonstrated at biochemical and molecular levels (1-3), the 'Warburg' hypothesis of mitochondrial function impairment (4) has not been established in cancer biology. In fact, some 80 years later, we continue to seek biochemical and molecular biology evidence of mitochondria's role, and its extent, in neoplastic transformation and in maintenance and/or promotion of neoplasia. Over the past two decades, our energy reactors have taken a center stage at another process central to neoplasia – programmed cell death.

Following Andrew Wylie's 1972 observation on the significance of natural cell death in mammary gland tissue, coined apoptosis, efforts to understand its molecular effectors have taken a center stage in countless laboratories around the world. Today, we know that apoptosis is an 'active' process indispensable to support of normal physiological processes from embryonic development to maintenance of tissue homeostasis via preservation of genomic and phenotypic integrity. If the cell(s) is damaged beyond repair (i.e. xenobiotic-induced toxicity), intrinsic apoptotic pathway is activated. If the outside system (i.e. tissue, immune surveillance) deems the cell(s) undesirable, extrinsic pathway is activated. Regardless of whether intrinsic or extrinsic, the sequence of events can be summarized as: Signal Initiation, Cellular Apoptosis Commitment, and Apoptosis Execution. Finally, the cell's remnants are packaged into vesicles (apoptotic bodies) and recycled by its microenvironment. Each of the three stages is regulated by a different subset of molecular effectors with different localization. According to prevailing current evidence, mitochondrial level signaling cascades constitute the 'Commitment' stage – mitochondria decide whether the cell dies or

not. Specifically, release of cytochrome c from the mitochondrial intermembrane space is generally accepted to be the molecular point of no return.

In molecular cancer research, multiple lines of evidence and reason suggest that “one renegade cell” must evade a program entrusted with maintenance of genomic and phenotypic integrity, in order to uncontrollably divide and ultimately conquer the host, remarkably similar to our early ancestors - the bacteria. In addition to the central role in early carcinogenic events, further dysregulation of apoptotic signaling cascades is strongly implicated in phenomenon of multi-drug resistance. **If Warburg was correct, and mitochondrial dysregulation is a defining feature of cancer, as supported by autonomy via apoptosis inactivation (gain of function), is there a molecular rationale (i.e. signaling link) to apparent loss of function via reliance on net-energy inefficient glycolysis amidst massive energy needs?** This was a central question preoccupying most of the cerebral bandwidths of a young graduate student, yours truly, embarking onto his PhD journey. What experimental model could shed light on the link? Enter Chromium (VI).

1.2. Chromium exposure

Hexavalent chromium [Cr(VI)] compounds are known environmental contaminants and human occupational respiratory carcinogens mostly originating from industrial processes (5). While the primary route of exposure is inhalation, additional modes include oral ingestion via contaminated water, or by direct dermal contact with products manufactured using chromium (6). The environmental release of chromium is estimated at over 90,000,000 pounds annually (7). For example, the burning of one cigarette releases up to 0.5 µg of chromium. It is estimated that the atmospheric concentration of particulate chromates in rural or residential areas of the U.S. ranges from 0.2 to 9 ng/m³ (7). In urban areas, especially in areas with a ferrochrome industry presence, those concentrations can easily be 10–100 fold higher (8). If one assumes that an

urban dweller in a city with a ferrochrome production facility receives an inhalation exposure of 20 m³ per day of 90 ng/m³, then the daily lung intake is 1.8 µg/day. Epidemiological studies carried out in the U.K., Europe, Japan and the U.S. have consistently shown that workers in occupations where particulate chromates are generated or used have an elevated risk of respiratory disease including lung cancer (9,10). Chromate workers have been found to have marked lung burdens of chromium with lung concentrations up to and exceeding 10,000 µg chromium per 100 g wet weight (11,12). Taken together, extensive studies and data collection on the industrial and environmental exposure to chromium compounds have led the National Institute of Occupational Safety and Health (NIOSH) to list chromate compounds as one of the major causes of occupational lung cancer, and the U.S. Environmental Protection Agency (U.S. EPA) and the International Agency for Research on Cancer (IARC) to classify chromium as a human carcinogen, currently making Cr(VI) one of 33 compounds listed to pose the greatest potential health threat in urban areas (6,13–15).

1.3. Chromium metabolism

Chromium is a transition-group metal that has several oxidation states (6). Under physiological pH and temperature, Cr(VI) is unable to interact with DNA and lacks genotoxic activity (20). The structural similarity of chromates to physiological sulfates and phosphate ions, enables molecular mimicry and easy entry of Cr(VI) into cells through non-specific anion channels (Fig. 3) (19,21). Cr(VI) enters the cell as an oxyanion followed by its metabolic reduction to Cr(V), Cr(IV), and to the final reduced trivalent (III) form. These reduced forms have been shown to induce a wide range of genomic DNA damage, which may lead to DNA replication inhibition (22). At physiological pH, intracellular reduction is facilitated by several molecules/compounds such as ascorbate, glutathione (GSH), cysteine, lipoic acid, hydrogen peroxide, NAD(P)H, fructose and ribose (16,18-20,23). The respective cellular availability and reaction rate with Cr(VI) determines which is the primary reducing agent. GSH, which is highly abundant

intracellularly and found at millimolar concentrations, has been identified as a modulator of cell stress induced by Cr(VI) cytotoxicity. Upon GSH depletion, Cr(VI) induces an overexpression of heme-oxygenase 1 in human dermal fibroblasts, which has been used as a marker for Cr(VI)-induced cell stress and cytotoxicity (24). The final product of chromate reduction, Cr(III), is biologically stable and has a greater DNA binding efficiency than that of Cr(VI) (16). Notably, extracellular reduction of Cr(VI) to Cr(III) detoxifies the molecule due to the inability of Cr(III) to cross the cellular membrane. However, when Cr(VI) is reduced in the cell to Cr(III), the weak membrane permeability of the trivalent form intracellularly traps the molecule, enabling the formation of stable Cr(III) complexes with proteins and nucleic acids leading to a large spectrum of DNA damage (19).

1.4. Chromium-induced oxidative stress

The reduction of Cr(VI) has been shown to yield reactive species such as free radicals, superoxide anions and hydroxyl radicals, possibly through Fenton-like reactions of Cr(V) and Cr(IV) with hydrogen peroxide (18,25). Several electron spin resonance (ESR) spin trapping studies have shown the generation of hydroxyl radicals in conjunction with long-lived Cr(V) species, following the reduction of Cr(VI) by NADPH and glutathione (25–35). While the direct relationship between DNA-reactive oxygen species and chromium-induced DNA damage is heavily debated and unclear, there have been several studies supporting the role of ROS in Cr(VI)-induced genotoxicity, cytotoxicity, and oxidative stress (17,35–39). To date, many studies have attempted to investigate the complexities of Cr(VI)-mediated oxidative stress and damage. Sugiyama et al. have shown Cr(VI) can be reduced to Cr(V) in the presence of vitamin B2 (riboflavin) in Chinese hamster V79 cells, leading to an increase in hydroxyl radicals, chromosomal aberrations and mutations at the HGPRT locus (37,40). Moreover, when pretreated with flavin adenine dinucleotide or vitamin E (a free radical scavenger), there was a marked decrease in Cr(VI)-induced single-strand

breaks and cytotoxicity (41). Other in vitro studies have shown that antioxidant regulation may be dependent on the concentration of Cr(VI). Asatiani et al. demonstrated in human epithelial like L-41 cells and fetal human lung fibroblasts, that toxic Cr(VI) concentrations (20 μ M) lead to an increase in ROS, and a significant reduction in catalase, glutathione, and cytosolic Cu/Zn superoxide dismutase activity (42). In contrast, there was a marked increase in catalase and glutathione peroxidase activity following non-toxic doses of Cr(VI) (2 μ M), demonstrating the existence of thresholds to Cr(VI)-induced oxidative stress. Furthermore, many reports studying Cr(VI)-mediated oxidative stress investigate its effect on cell death, or apoptosis. A study by Shi et al. suggests that ROS contributes to the early effects of Cr(VI)-induced apoptosis via a p53-independent mechanism, whereas the apoptotic effects could be blocked in the presence of ROS scavengers, such as catalase, aspirin and N-acetyl-L-cysteine (35). Bagchi et al. have also shown an increase in ROS production that correlates with apoptotic death in a Cr(VI) concentration and dose-dependent fashion (43). Although there are many studies reporting on the relationship between oxidative stress and Cr(VI)-mediated cytotoxicity, genotoxicity, and potential carcinogenesis, due to non-uniform treatment protocols, conflicting data and controversial methods used for detection of Cr(VI)-induced oxidative stress, this relationship remains unclear. Martin et al. demonstrated that the high valence chromium species, bis(2-ethyl-2-hydroxybutyrate) oxochromate (V) [Cr(V)-EHBA], was able to independently induce the fluorescence of two dyes commonly used to detect ROS (2,7-dichlorofluorescein and dihydrorhodamine) in A549 cells, which was not affected by treatment with radical scavengers (44). These data suggest that 2,7-dichlorofluorescein and dihydrorhodamine are more suitable for the qualitative detection of Cr(V), rather than ROS production in the presence of Cr(VI). Moreover, it is likely that any discussion on the potential role of Cr(VI)-induced oxidative stress needs to include careful consideration of total dose over time and dose rate, as well as the relative ratios and availability of intracellular reductants and anti-oxidants. Given the intricate and unclear relationship between apoptosis and oxidative stress, it will be very difficult to determine

whether Cr(VI)-associated oxidative stress is caused by, or causes, the initiation of apoptosis.

1.5. Cr(VI)-induced DNA damage and mutagenesis

Chromium-induced DNA damage is thought to be the primary mechanism of chromate genotoxicity and mutagenicity, but it is only clearly observed at doses that are also capable of producing cell death (45). Recently, data has been presented to the EPA's Cancer Assessment Review Committee (CARC) to support mutagenicity as the initiating step in Cr(VI)-induced carcinogenesis (46). Structural genetic lesions produced by Cr(VI) include DNA adducts, DNA-strand breaks, DNA-protein crosslinks, oxidized bases, abasic sites, and DNA inter- and intrastrand crosslinks (20,47). The association of chromium with the phosphodiester backbone of DNA (chromium-DNA adduct) is one of the most abundant genetic lesions induced by chromium in mammalian cells and is thought to be a primary cause of Cr(VI) mutagenicity (19). Cr(III)-ligand-DNA complexes form binary and ternary adducts, the latter of which are predominant and more toxicologically relevant (19,20). There are four types of ternary adducts that primarily persist following Cr(VI) exposure and intracellular reduction; they are Cr(III)-ascorbate-, Cr(III)-cysteine-, Cr(III)-histadine-, and Cr(III)-glutathione-DNA adducts, respectively (19).

Mutational analysis studies using SV-40 immortalized human fibroblasts transfected with plasmids extracellularly treated with Cr(VI), showed that the primary and most mutagenic adduct formed was ternary in nature, and that the Cr(III)-cysteine-DNA and Cr(III)-ascorbate-DNA adducts were more mutagenic than the binary adducts (48,49). O'Brien et al. showed significant DNA damage in the form of DNA-chromium-DNA crosslinks and DNA-chromium-GSH crosslinks following treatment of DNA with Cr(III) and Cr(VI) with ascorbate and GSH, respectively (22). The damage induced by Cr(VI) can lead to dysfunctional DNA replication and transcription causing base substitutions and deletions

serving as substrates for base excision repair (BER) and nucleotide excision repair (NER), and promoting genomic instability (50). Genomic instability, which can be caused by aberrant cell cycle checkpoints and dysregulated DNA repair mechanisms via microsatellite instability (MIN), plays an important role in Cr(VI) carcinogenesis (48). There are several lines of evidence suggesting the importance of base substitutions in Cr(VI) mutagenicity, with G:C substitutions shown to be the primary target in vitro and in vivo; however, studies supporting the relevance of specific Cr(VI)-induced base deletions are inconsistent (48). Furthermore, mechanistic studies in Cr(VI)-induced toxicity and mutagenesis are lacking a focal point of mitochondria.

FIGURE 1. Schematic Representation of Cellular Energy Metabolism.

FIGURE 2. Diagrammatic Illustration of Oxidative Phosphorylation Pathway.

FIGURE 3. Schematic representation of known cellular reduction and cytotoxic effects of Cr(VI).

1.6. Summary

A. Sustained environmental exposure to Cr (VI) compounds leads to a multitude of respiratory illnesses including lung cancer. Lung cancers mostly manifests as epithelial cell derived carcinomas. The process by which chromium is able to induce neoplastic progression is both complex and fairly elusive. Data can be found on each end of the spectrum, either supporting or refuting the many mechanisms proposed in the involvement of Cr(VI) carcinogenesis. Given phenotypic tissue diversity and considerable controversy surrounding observations on Cr(VI)-mediated toxicity and mutagenesis, it is still unknown whether, and to what extent, human lung epithelial cells are susceptible to Cr(VI)-induced toxicity and carcinogenesis. This is largely due to the lack of an appropriate model system to study the molecular mechanisms involved in Cr(VI)-induced malignant transformation and carcinogenesis.

B. It is well established that the spectrum of cellular effects of Cr(VI) begins with its intracellular metabolism, reactive oxygen species production, formation of Cr–DNA adducts, genomic damage, and alteration of survival signaling pathways. The causal early mechanistic events are accepted to be ‘chemical’ in nature, a function of Cr and O₂ chemistry. It is of no surprise therefore that little is known about the role of mitochondrial physiology in cell’s ultimate fate.

C. Mitochondria lie at the center of two cellular processes fundamental to evolution and maintenance of neoplasia – energy production and apoptosis. The potential causal relationship between the two processes has proven to be elusive despite rigorous scientific pursuit for almost 2 decades. The Warburg Paradox remains one of the central questions in molecular cancer research.

1.7. Specific Aims

Although Cr(VI) is an established human carcinogen, its mechanism of action remains unclear. The overall objective of this study was to determine the molecular mechanisms of Cr(VI)-induced apoptosis and malignant transformation with the goal of improving our understanding on Cr(VI)-induced carcinogenesis. To achieve this goal we accomplished the following specific aims:

Aim I: Establish a lung cancer relevant model to study the mechanisms of Cr(VI)-induced toxicity and carcinogenesis.

Aim II: Identify molecular effectors underlying the cellular response to Cr(VI)-induced stress with the focal point of mitochondria.

Aim III: Deduce experimental basis for reconciliation of the Warburg Paradox.

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CHAPTER II

**Energy Substrate Modulates Cr(VI)-Induced Apoptosis
Differentially in Cancerous vs. Non-Cancerous cells via a
Mitochondrial Reactive Oxygen Species-Dependent Mechanism**

2.1 Abstract

Cr(VI) compounds are redox cycling carcinogens that induce apoptosis as a primary mode of cell death. Since defects in apoptosis regulatory mechanisms are considered to be indispensable foundation of carcinogenesis it is critical to understand the molecular effectors involved. Cellular energy powerhouses, the mitochondria, house the molecular effectors involved in signaling cascades comprising commitment to apoptosis and are reported to be dysregulated in a variety of tumors. The objective of this study was to characterize the key signaling effectors in Cr(VI)-induced apoptosis and determine the relationship between those effectors and mode of cellular energy production in cancerous vs. non-cancerous cells. Our data show that Cr(VI) induces mitochondrial ROS dependent apoptosis in human lung cancer (H460) and non-cancer (BEAS-2B) cells. The intensity of mitochondrial ROS production and consequently apoptosis is highly dependent on the energy substrate available to the cells. Forcing of oxidative phosphorylation-only energy metabolism abrogates native 4-fold apoptosis resistance in cancerous H460 cells vs. non-cancerous BEAS-2B cells. Collectively, our report presents a mechanistic advancement in the understanding of molecular effectors underlying the Warburg's "mitochondrial dysregulation" hypothesis and puts mitochondrial ROS as key mediators of the commitment phase of programmed cell death in a model of Cr(VI)-induced toxicity.

2.2 Introduction

Mitochondria play an essential role in providing energy to the eukaryotic cell. In recent years, mitochondria have become a central subject of study because of their role as sensors and executioners of apoptosis (1–3). Apoptosis is an energy-dependent, genetically encoded program for cell death that is indispensable for the normal development of the organism (4). Alterations in the cellular program of apoptosis contribute to the progression of various human pathologies, including cancer and neurodegenerative diseases (5, 6). The study of the energy metabolism of cancer cells was a central issue of cancer research until the era of molecular biology. As early as 1930, Otto Warburg proposed the hypothesis that cancer cells may have impaired mitochondrial function and that this alteration would result in the elevated rate of glycolysis that is a common feature of most tumors (7). Although the glycolytic phenotype of many cancer cells and tumors has been demonstrated at both the biochemical and molecular levels (8 –10), the presumed impairment of mitochondrial function was never established in cancer biology (11). Differences in the number of mitochondria (12), their ultrastructure (13, 14), the content and composition in all OXPHOS complexes (12, 15–18), respiratory chain activity (18, 19), the expression of oxidative phosphorylation dependent genes (20), and levels of mitochondrial DNA (15) have all been variously reported by different investigators, but no consistent pattern has emerged. When glucose is no longer available, as can occur in solid tumors (21), cancer cells are forced to use alternative energy substrates such as the oxidation of glutamine, a process called glutaminolysis (22). This process requires an active oxidative phosphorylation for ATP production. Here, we have examined the physiological effects of such a change in substrate availability on a human lung cancer cell line (H460) vs. non-cancer cell line (BEAS-2B) in a model of Cr(VI)-induced acute toxicity.

Inhalation of Cr(VI) compounds as dust particles and fumes is a common form of exposure that can cause multiple respiratory effects including lung cancer (23,

24,25–28). More than 300,000 workers in the United States are potentially exposed to Cr(VI) and Cr(VI)-containing compounds in the workplace. Cr(VI) enters cells through a non-specific anion transport system (29). Once inside, it is reduced by cellular reductants to its lower oxidation states, pentavalent chromium (Cr(V)) and tetravalent chromium (Cr(IV)) (30). The lower oxidation intermediates of chromium are reactive and lead to production of reactive oxygen species (ROS) (31, 32) which, in turn, cause various levels of oxidative damage including DNA strand breaks, base modification and lipid peroxidation (30, 33–37). Acute laboratory exposure generally results in cell death in the form of apoptosis (38) or mutagenesis (39) depending largely on Cr(VI) dose and the system used.

To date, specific molecular effectors constituting cellular response to acute Cr(VI) stress remain poorly understood, especially at the level of mitochondria. Furthermore, the effect of energy substrate modulation (glycolysis vs. oxidative phosphorylation) has not been investigated in published Cr(VI) studies. Our study is the first comprehensive analysis of the effect energy substrate plays in Cr(VI)-induced acute toxicity in cancerous vs. non-cancerous cells. We identify mitochondrial ROS as the primary agents of commitment phase of programmed cell death and establish their quantitative dependence on cellular energetic mode. Further, forcing of oxidative phosphorylation-only energy metabolism abrogates native 4-fold apoptosis resistance in human lung cancer H460 cells vs. their non-cancerous BEAS-2B counterparts. Collectively, our report presents a mechanistic advancement in understanding of molecular effectors underlying the Warburg's "mitochondrial dysregulation" hypothesis and establishes a model for further mechanistic studies of xeno-biotic toxicities.

2.3 Materials and Methods

Chemicals and Reagents

N-acetyl cysteine (NAC), rotenone, diphenylene iodonium (DPI), sodium pyruvate, buthionene sulfoximine (BSO), Tris-HCl, Triton X-100, NaCl, glycerol, Na₃VO₄, NaF, phenylmethylsulfonyl fluoride (PMSF), bicinchoninic acid (BCA), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), monochlorobimane (mBCl), glucose and sodium dichromate (Na₂Cr₂O₇·2H₂O) [Cr(VI)] were obtained from Sigma Chemical Inc. (St. Louis, MO). The spin trap DMPO was purified by charcoal decolorization and vacuum distillation. DMPO solution, thus purified, did not contain any electron spin resonance (ESR)-detectable impurities. Phosphate-buffered saline (PBS) and RPMI-1640 medium were purchased from Gibco BRL (Gaithersburg, MD). Chelex 100 chelating resin was purchased from Bio-Rad Laboratories (Richmond, CA). The phosphate buffer (pH 7.4) was treated with Chelex 100 to remove transition metal ion contaminants. Hanks balanced salt solution (HBSS) was purchased from Mediatech (Herndon, VA). Defined fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Apoptosis dye Hoechst 33342 was purchased from Molecular Probes (Eugene, OR). Antibodies for glutathione peroxidase, (GPx), manganese superoxide dismutase (MnSOD), Bcl-2, peroxidase-labeled secondary antibodies, anti-myc agarose beads, and protein A-agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Transfecting agent Lipofectamine 2000 was from Invitrogen (Carlsbad, CA).

Cell Culture

The human lung bronchial epithelial BEAS-2B and human lung epithelial cancer H460 cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) containing 5% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin in a 5% CO₂, standard atmosphere environment at 37°C. Cells were passaged at preconfluent densities using a solution containing 0.05% trypsin and 0.5 mM EDTA (Invitrogen). Energy substrate modulation

studies were conducted in RPMI glucose-free medium containing 20 mM pyruvate and 10 mM L-glutamine. The control media contained 20 mM glucose instead of pyruvate. Cells were acclimated to the media at least 24 h prior to the commencement of the experiments.

Apoptosis Assay

After specific treatments, apoptosis was determined by incubating cells with 10 µg/ml Hoechst 33342 nuclear stain for 30 min at 37°C and scoring the percentage of cells having intensely condensed chromatin and/or fragmented nuclei by fluorescence microscopy (Axiovert100, CarlZeiss) using Pixera software.

Mitochondrial Membrane Potential ($\Delta\psi_m$)

$\Delta\psi_m$ was estimated using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole carbocyanide iodide (JC-1; Molecular Probes). JC-1 is a fluorescent compound (excitation maximum, 490 nm) that exists as a monomer at low concentrations. At higher concentrations, JC-1 forms aggregates. Fluorescence of the monomer is green (emission, 527 nm), whereas that of the J aggregate is red (emission, 590 nm). Mitochondria with intact membrane potential (higher than 100 mV) concentrate JC-1 into aggregates that fluoresce red, whereas de-energized mitochondria cannot concentrate JC-1 and fluoresce green. Cells were grown in 25-cm² dishes until 90% confluence and either kept in the medium alone (control) or treated with 1 mM Cr(VI) for 1 h. Cells were washed with PBS and incubated at 37°C for 45 min with 6.5 mM JC-1 in media in the dark. Cells were collected by trypsinization, washed in PBS, and resuspended in 500 µl of PBS. The ratio of the reading at 590 nm to the reading at 527 nm (590:527 ratio) was considered as a relative $\Delta\psi_m$ value.

Stable Transfection

Stable transfectants of glutathione peroxidase (GPx), manganese superoxide dismutase (MnSOD) and Bcl-2 were generated by culturing BEAS-2B and H460

cells in six-well plates until they reached 80% confluence. 1 μ g of cytomegalovirus -neo vector and 15 μ l of Lipofectamine 2000 reagent with 2 μ g of Bcl-2, GPx, MnSOD, or control pcDNA3 plasmid were used to transfect the cells in the absence of serum. After 10 h, the medium was replaced with culture medium containing 5% fetal bovine serum. Approximately 36 h after the beginning of the transfection, the cells were digested with 0.03% trypsin, and the cell suspensions were plated onto 75 ml culture flasks and cultured for 24 to 28 days with G418 selection (400 μ g/ml). Stable transfectants were identified by Western blot analysis and were cultured in G418-free RPMI 1640 medium for at least two passages before each experiment.

Electron Spin Resonance ROS Assay

Spin trapping was used to detect short-lived free radical intermediates. This technique involved the addition-type reaction of a short-lived radical with a paramagnetic compound (spin trap) to form a relatively long-lived free radical product (spin adduct), which was then studied using conventional ESR. All ESR measurements were conducted using a Bruker EMX spectrometer (Bruker Instruments, Inc., Billerica, MA) and a flat cell assembly. The intensity of the signal was used to measure the amount of short-lived radicals trapped; the hyperfine couplings of the spin adduct were generally characteristic of the original trapped radicals. Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) as reference standards. The relative radical concentration was estimated by multiplying half of the peak height by $[\Delta H_{pp}]$, where ΔH_{pp} represents peak-to-peak width. An Acquisit program was used for data acquisition and analysis. BEAS-2B or H460 cells were mixed with DMPO (100 mM) and PBS to a final volume of 1 ml. The mixtures were then incubated for 5 min in a 37°C water bath. The reaction mixture was then transferred to a flat cell for ESR measurement. Experiments were performed at room temperature under ambient air.

Western Blotting

After specific treatments, cells were incubated in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM Na₃VO₄, 50 mM NaF, 100 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor mixture for 20 min on ice. After insoluble debris was precipitated by centrifugation at 14,000 x g for 15 min at 4°C, the supernatants were collected and assayed for protein content using bicinchoninic acid (BCA) method. Equal amount of proteins per sample (15 µg) were resolved on 10% SDS-PAGE and transferred onto a 0.45-µm nitrocellulose membrane. The transferred membranes were blocked for 1 h in 5% non-fat dry milk in TBST (25 mM Tris-HCl, pH 7.4, 125 mM NaCl, 0.05% Tween 20) and incubated with the appropriate primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The immune complexes were detected by chemiluminescence (Supersignal, West Pico; Pierce).

Statistical Analysis

The data were expressed as means ± SD of three or more independent experiments. Statistical analysis was performed using two-tailed and paired Student's t-test. P values less than 0.05 were considered statistically significant and indicated by an asterisk

2.4 Results

Cr(VI)-Induced Cell Death

Cr(VI) induces apoptosis as the primary mode of cell death during acute laboratory exposure (40). Dysregulation of apoptosis regulatory mechanisms is widely accepted to be one of the hallmarks of cancer (41). To characterize the apoptotic response to Cr(VI) treatment in our system, non-tumorigenic human bronchial epithelial (BEAS-2B) cells and human lung cancer (H460) cells were treated with different doses of Cr(VI) (0-50 µM) and apoptosis was determined after 12 h by an established Hoechst 33342 assay. Figs. 1, A and B, show that

Cr(VI) treatment caused a dose dependent increase in cell apoptosis over control level, as indicated by increased nuclear fluorescence and chromatin condensation of the treated cells. Notably, human lung cancer H460 cells showed increased resistance to Cr(VI)-induced apoptosis vs. their non-tumorigenic counterparts. At 20 μ M Cr(VI), an approximate LD₅₀ for BEAS-2B, human lung cancer H460 cells exhibited approximately 10% apoptotic response.

Energy Substrate Effect on Cr(VI)-Induced Cell Death

Mitochondrial level signaling has been implicated in Cr(VI)-induced apoptosis. Various reports suggest that mitochondria are dysregulated in cancer cells and that cancer cells prefer to synthesize energy via less net-energy efficient glycolysis as opposed to mitochondria-localized oxidative phosphorylation. Accordingly, the effect of energy substrate on Cr(VI)-induced cell death was evaluated in BEAS-2B and H460 cell populations grown in glucose containing (G+P-) or glucose lacking, pyruvate containing (G-P+) medium. Figs. 2, A and B, show that energy substrate modulation significantly altered Cr(VI)-induced toxicity profile in both, BEAS-2B and H460 cells. BEAS-2B cells became approximately 50% more sensitive to Cr(VI) 20 μ M dose under energy substrate conditions forcing oxidative phosphorylation only metabolism. Notably, cancer H460 cells' sensitivity increased approximately 400% when oxidative phosphorylation was forced, resulting in approximately 8-fold increase in apoptosis and quantitative lethal dose normalization between the two cell types (Fig. 2C).

Effect of Cr(VI) on ROS Generation

Reactive oxygen species (ROS) have been strongly implicated in mechanism of Cr(VI)-induced toxicity. We deployed ESR spin trapping to determine the identity and intensity of free radical generation in Cr (VI)-stimulated BEAS-2B and H460 cells. Figs. 3A-B show the typical ESR spectra obtained from a mixture containing 10⁶ cells/ml, Cr(VI) and 100 mM spin trap (DMPO) in a phosphate buffer solution (pH 7.4). The spectrum exhibits a 1:2:2:1 quartet with hyperfine

splittings of $a_H = a_N = 14.9$ G, where a_H and a_N denote hyperfine splittings of β -hydrogen and nitroxyl nitrogen, respectively. Based on these splitting constants (42), this quartet spectrum was assigned to DMPO/ $\cdot$ OH adduct, which is an indirect evidence for $\cdot$ OH generation. Cells alone did not generate any observable ESR signal. The weak signal at the right side is Cr (V), which was generated during the Cr (VI) reduction by the cells. Figure 3C shows spectral intensity (amount of $\cdot$ OH radicals generated) in BEAS-2B vs. H460 cells. Notably, non-tumorigenic BEAS-2B cells showed significantly stronger radical generation than their cancer H460 counterparts across the 0.1–1 mM Cr(VI) range at 10 min post-treatment. Consistent with previously observed (43) inverse relationship between toxic stimuli initiated cellular ROS production and $\Delta\psi_m$, we observed a dose dependent decrease of $\Delta\psi_m$ in both cell lines (Fig. 3D) 2 h post Cr(VI) treatment. Notably, cancer H460 cells showed approximately 50% lower $\Delta\psi_m$ as compared to non-tumorigenic BEAS-2B cells.

Effect of Energy Substrate on Cr(VI)-Induced ROS Generation and $\Delta\psi_m$

ROS have been demonstrated to play a critical role in Cr(VI)-induced cell death and are detected in our system. Their origin, however, is subject to considerable controversy. In order to determine the relative role of oxidative phosphorylation system on radical production in our model, we utilized ESR spectroscopy to determine qualitatively and quantitatively the effect of energy substrate on Cr(VI)-induced radical production. Figs. 4A-B show 1:2:2:1 quartet with hyperfine splittings consistent with $\cdot$ OH production, under conditions favoring glycolysis and, those forcing oxidative phosphorylation, in both cell types. Notably, non-tumorigenic BEAS-2B cells cultured in the medium with no glucose, showed no significant difference in spectral intensity whereas cancer H460 cells exhibited significantly different response to forced oxidative phosphorylation with approximately 40% increase in spectral intensity (Fig. 4C). Both cell lines exhibited a slight increase in $\Delta\psi_m$ in glucose lacking medium (Fig. 4D).

Effect of ROS Modulators on Cr(VI)-Induced Apoptosis

To establish a causal relationship between the observed radical production and apoptosis following the Cr(VI) insult, we turned to well established small molecule inhibitors and inducers of ROS. Figs. 5A-C suggest a causal role of ROS in Cr(VI)-induced apoptosis. Pretreatment of the cells with L-buthionine-S,*R*-sulfoximine (BSO), an oxidative stress inducer (44), significantly increased the apoptotic effect of Cr(VI) in both BEAS-2B and H460 cells. Of interest, H460 apoptosis increased approximately 300% whereas BEAS-2B cells showed significant, but relatively marginal increase of 30%. Pretreatment with N-acetylcysteine (NAC), a widely accepted, broad spectrum antioxidant, resulted in a complete apoptosis shutdown with proportion of Hoechst 33342 positive cells returning to control levels in both cell types.

Source of ROS Involved in Cr(VI)-Induced Apoptosis

To assess the relative importance of mitochondrial vs. cytosolic ROS on Cr(VI)-induced apoptosis, cells were treated with Cr(VI) in the presence or absence of diphenylene iodonium (DPI), a specific inhibitor of NADPH oxidase (45, 46), or rotenone, a mitochondrial electron transport chain interrupter (47, 48), and their effects on system apoptosis were examined. The results show that both rotenone and DPI reduced Cr(VI)-induced apoptosis, with the effect of rotenone being much larger (Figs. 6, A-B). These results suggest that even though cytosolic ROS may be involved, mitochondrial oxidative phosphorylation chain seems to generate the majority of ROS involved in Cr(VI)-induced apoptosis.

Effect of Glutathione Peroxidase and Superoxide Dismutase on Cr(VI)-Induced Apoptosis

To confirm the role of mitochondrial ROS in Cr(VI)-induced apoptosis by means of genetic manipulation, both cell types were stably transfected with endogenous antioxidant enzyme GPx, MnSOD, or control plasmid, and their effects on apoptosis were determined. GPx and MnSOD are the two primary enzymes charged with detoxifying ROS emanating from the electron transport chain of mitochondria and are localized in the mitochondrial matrix (49-52). Notably,

apoptosis assay showed a significant decrease in apoptotic response to Cr(VI) treatment in GPx- and MnSOD-transfected cells but not in mock-transfected cells (Figs. 7A-C). These observations suggest that aside from the already established role of hydroxyl radical ($\cdot\text{OH}$), hydrogen peroxide (H_2O_2) and superoxide anion ($\cdot\text{O}_2^-$) play critical roles in Cr(VI)-induced apoptosis.

Effect of Bcl-2 on Cr(VI)-Induced Apoptosis

To determine whether elevated mitochondrial ROS production represents a commitment point in Cr(VI)-induced apoptosis, localized to mitochondrial matrix, we created stable clones overexpressing Bcl-2 gene. Bcl-2 has been studied extensively and its role in apoptosis regulation at the level of mitochondria is well established. Specifically, it localizes to the outer mitochondrial membrane where it inhibits a multitude of pro-apoptotic signaling events (53, 54). Mutants stably overexpressing Bcl-2, in both cell types, showed significant reduction in Cr(VI)-induced apoptosis (Figs. 8A-B). Notably, the relative intensities of apoptosis reduction were comparable to observations in GPx and MnSOD overexpressing clones, suggesting additional signaling cascades between mROS production and outer mitochondrial membrane signaling events.

2.5 Discussion

In order to survive, cells must respond to changes in environment. The adaptability of our cellular systems is considerable, but not unlimited. Nowhere is that more evident than in neoplasia. The Warburg effect, or increased dependency on glycolysis in cancer cells, has been a long-standing observation (7-9). Despite numerous experimental findings of this metabolic alteration in a wide spectrum of human cancers, the mechanisms underlying it and potential therapeutic implications still remain a subject of considerable controversy (8-10). The central question is whether a shift to glycolysis is **simply an adaptation** due to low availability of oxygen in tumor microenvironment (Figure 9, a majority opinion), or, a consequence of “respiratory system injury”, as Warburg himself

thought, **forcing** the cells to use the glycolytic pathway to generate ATP. While multiple lines of evidence in the literature support both contentions, several fundamental challenges face “the majority opinion”. First, cancer cells maintain the glycolytic phenotype in cell culture where oxygen is **freely** available. Second, the contention that once adaptation to glycolysis occurs, it becomes irreversible, is not supported by evidence. Contrary, several reports highlight significant plasticity of mitochondria under changing metabolic conditions (11-13). If mitochondria are so energy substrate versatile, and produce 36 ATP molecules per glucose unit vs. 2 ATPs from glycolysis, what benefit would outweigh the tremendous net energy cost? Collectively, our data point toward ROS (Figure 10).

Cr(VI) is a known human carcinogen that has been associated with lung cancer (25). Its cytotoxic and mutagenic effects have been studied extensively and point toward ROS as causal in Cr(VI)-induced toxicity and mutagenesis. Indeed, our data show a dose dependent increase in apoptosis in non-tumorigenic BEAS-2B cells as well as tumorigenic H460 cells. H460 cells, however, are approximately 4 fold more resistant to Cr(VI)-induced apoptosis than their non-tumorigenic counterparts (Figs. 1A-B). When exposed to a functioning oxidative phosphorylation-dependent, energy substrate-only, environment, the observed apoptosis resistance is abrogated (Figs. 2A-C). This is the first report of energy substrate modulation of Cr(VI)-induced toxicity indicating a link between oxidative phosphorylation only metabolism and mechanism of Cr(VI)-induced toxicity. Furthermore, observations in Fig. 2 suggest critical ‘differences’ between H460 and BEAS-2B cells in support of Warburg’s “respiratory defect”. Given the observations in Figure 2, we focused on delineating deeper mechanistic aspects of Cr(VI)-induced apoptosis in our system.

Cr(VI) is actively transported into cells by anionic transport system at the cellular level (55) and is metabolically converted to the ultimate reduced form, Cr(III), in a process that also produced ROS. Since generation of ROS is a consequence of

Cr(VI) exposure, we also demonstrated the role of ROS in Cr(VI)-induced apoptosis of non-tumorigenic BEAS-2B and tumorigenic H460 cells. Consistent with the observation that reduction of Cr(VI) inside the cell produces ROS, our results showed that Cr(VI) was able to induce $\cdot\text{OH}$ radical generation in both cell types (Figs. 3A-B). The relative intensity of the ESR spectra was significantly lower in the tumorigenic H460 cells (Fig. 3C) at both concentrations studied. Decrease in mitochondrial membrane potential has been linked to mitochondrial ROS production in response to toxic stimuli (11, 13) as well as overall mitochondrial function (15). This was the case in our system as well where we observed a dose dependent decrease in mitochondrial membrane potential in both cell types (Fig. 3D). The relative decrease was significantly higher in non-tumorigenic BEAS-2B cells.

In order to establish the link between Cr(VI)-induced ROS production and functioning mitochondrial metabolism, we evaluated the relative ESR peak intensity as a function of energy substrate available to the cells. Figures 4A-C show that conditions forcing oxidative phosphorylation resulted in a significant increase in ROS production in tumorigenic H460 cells, whereas BEAS-2B cells showed no significant difference. Consistent with the observed energy substrate effects on ROS production, we observed a significant increase in the mitochondrial membrane potential in both cell types (Fig. 4D) when oxidative phosphorylation was forced. Since we observed the correlation between energy substrate, apoptosis and ROS production, we set out to establish the potential causal relationship between the observed Cr(VI)-induced ROS and apoptosis. Increase in ROS production was achieved via pretreatment of the cells with an established ROS inducer BSO, while NAC was used as a ROS inhibitor. BSO pretreatment led to a significant increase in Cr(VI)-induced apoptosis in both cell types, while NAC virtually abrogated the effect (Figs. 5A-C), suggesting a causal relationship between the ROS and apoptosis in our system. This observation was consistent with previous reports on the role of ROS in Cr(VI)-induced apoptosis (29-31).

While ROS production is a known factor in Cr(VI)-induced toxicity, their sub-cellular origins are not well understood. To determine the source of ROS involved in Cr(VI)-induced apoptosis in our system, we utilized DPI and rotenone.

Rotenone has previously been shown to inhibit mitochondrial ROS while DPI is known to inhibit cellular NADPH oxidase; both are considered as indicators of the mitochondrial and cytosolic ROS, respectively (56-58). Cr(VI)-induced ROS generation and apoptosis was significantly inhibited by DPI and rotenone, with the effect of the latter being more dominant (Fig. 6A-B) in both cell types. This indicates that total ROS produced are important in Cr(VI)-induced apoptosis, but mitochondrial ROS play a major role in our system. DPI and rotenone are widely used in mechanistic studies; however, secondary effects cannot be excluded due to their small-molecule nature. In order to confirm the causal relationship mitochondrial ROS plays in Cr(VI)-induced apoptosis in our system, stable cell lines overexpressing GPx and MnSOD were created for both cell types. Gene transfection studies using GPx- and SOD-overexpressing cells confirmed that mitochondrial ROS play a crucial role in Cr(VI)-induced apoptosis (Figs. 7A-C). Apoptosis was virtually abrogated in both, the tumorigenic H460 as well as non-tumorigenic BEAS-2B mutants.

To determine whether mROS production in our system represents a point of no return for the cell in Cr(VI)-induced apoptosis, stable clones overexpressing Bcl-2 gene were created. Bcl-2 has been studied extensively and its role in apoptosis regulation at the level of mitochondria is well established. Specifically, it localizes to the outer mitochondrial membrane where it inhibits a multitude of pro-apoptotic signaling events (53, 54). Mutants stably overexpressing Bcl-2, in both cell types, showed significant reduction in Cr(VI)-induced apoptosis (Figs. 8A-B) comparable in intensity to the observations in GPx and MnSOD-overexpressing clones. Together, these observations suggest additional signaling cascades between mROS production in the matrix and outer mitochondrial membrane signaling events.

In summary, our data provide evidence that apoptosis induced by Cr(VI) is mediated through the mitochondria-dependent signaling pathway. ROS play a causal role in Cr(VI)-induced apoptosis of non-tumorigenic human bronchial epithelial BEAS-2B cells and human lung cancer H460 cells. The mechanism by which Cr(VI) induces apoptosis involves rapid generation of ROS in the mitochondrial matrix and consequent depolarization of the outer mitochondrial membrane. Further, mitochondrial ROS generation resulting from Cr(VI) insult is quantitatively dependent on the degree of cellular commitment to oxidative phosphorylation driven energy production. Forcing of oxidative phosphorylation-only metabolism leads to an abrogation of native apoptosis resistance in human lung cancer H460 cells vs. their non-tumorigenic BEAS-2B counterparts. Collectively, our data present a mechanistic advancement in the understanding of molecular effectors underlying the Warburg's "respiratory defect" hypothesis and put mitochondrial ROS as causal agents of programmed cell death in Cr(VI)-induced toxicity and carcinogenesis (Fig. 11).

Figure 1

FIGURE 1. Induction of apoptosis by Cr(VI) in human lung epithelial BEAS-2B and human lung cancer H460 cells. A, subconfluent monolayers of BEAS-2B and H460 cells were exposed to varying doses of Cr(VI) (0-50 µM) for 12 h, and the cells were analyzed for apoptosis by Hoechst 33342 assay. B, fluorescence micrographs of treated cells stained with Hoechst dye. Apoptotic cells exhibited shrunken and fragmented nuclei with bright nuclear fluorescence. Values are mean $\pm$ S.D. ($n > 3$). *, $p < 0.05$ versus non-treated control.

Figure 2

FIGURE 2. Effect of energy substrate on Cr(VI)-induced apoptosis in human lung epithelial BEAS-2B and human lung cancer H460 cells. A, subconfluent monolayers of BEAS-2B were exposed to 20 μ M dose of Cr(VI) for 12 h in either G+P- or G-P+ medium, and the cells were analyzed for apoptosis by Hoechst 33342 assay. B, subconfluent monolayers of H460 cells were exposed to 20 μ M dose of Cr(VI) for 12 h in either G+P- or G-P+ medium, and the cells were analyzed for apoptosis by Hoechst 33342 assay. C, Relative apoptosis resistance in H460 vs. BEAS-2B cells as a function of available energy substrate. Values are mean $\pm$ S.D. ($n > 3$). *, $p < 0.05$ versus non-treated control.

Figure 3

FIGURE 3. Cr(VI)-induced ROS production and mitochondrial membrane depolarization. *A*, BEAS-2B cells were exposed to varying concentrations of Cr(VI) (0.1 and 1 mM) and the cells were analyzed for ROS production using electron spin resonance (ESR) spectroscopy. *B*, H460 cells were exposed to varying doses of Cr(VI) (0.1 and 1mM) and the cells were analyzed for ROS production using ESR. *C*, Relative difference in the ROS intensity in BEAS-2B and H460 cells. *D*, Mitochondrial membrane depolarization as indicated by JC-1 fluorescence. Values are mean $\pm$ S.D. ($n > 3$). *, $p < 0.05$ versus non-treated control.

Figure 4

FIGURE 4. Effect of energy substrate on ROS production and mitochondrial membrane depolarization. A, BEAS-2B cells were exposed to 1 mM Cr(VI) and the cells were analyzed for ROS production using electron spin resonance spectroscopy (ESR) in G+P- and G-P+ media. B, H460 cells were exposed to 1 mM Cr(VI) and the cells were analyzed for ROS production using electron spin resonance spectroscopy (ESR) in G+P- and G-P+ media. C, Relative difference in the ROS intensity in BEAS-2B and H460 cells as a function of energy substrate. D, Relative difference in mitochondrial membrane depolarization as a function of energy substrate. Values are mean $\pm$ S.D. ($n > 3$). *, $p < 0.05$ versus BEAS-2B cells.

Figure 5

FIGURE 5. Effect of ROS modulators on Cr(VI)-induced apoptosis in BEAS-2B and H460 cells. A, subconfluent monolayers of BEAS-2B cells were pretreated for 1 h with BSO or NAC and exposed to 20 μ M Cr(VI) for 12 h, and the cells were analyzed for apoptosis by Hoechst 33342 assay. B, subconfluent monolayers of H460 cells were pretreated for 1 h with BSO or NAC and exposed to 20 μ M Cr(VI) for 12 h, and the cells were analyzed for apoptosis by Hoechst 33342 assay. C, fluorescence micrographs of treated cells stained with Hoechst dye. Apoptotic cells exhibited shrunken and fragmented nuclei with bright nuclear fluorescence. Values are mean $\pm$ S.D. ($n > 3$). *, $p < 0.05$ versus non-treated control. ** versus Cr(VI)-treated control.

Figure 6

FIGURE 6. Source of ROS involved in Cr(VI)-Induced Apoptosis *A*, subconfluent monolayers of BEAS-2B cells were pretreated for 1 h with ROT or DPI and exposed to 20 μ M Cr(VI) for 12 h, and the cells were analyzed for apoptosis by Hoechst 33342 assay. *B*, subconfluent monolayers of H460 cells were pretreated for 1 h with ROT or DPI and exposed to 20 μ M Cr(VI) for 12 h, and the cells were analyzed for apoptosis by Hoechst 33342 assay. Values are mean $\pm$ S.D. ($n > 3$). *, $p < 0.05$ versus non-treated control. ** versus Cr(VI)-treated control.

Figure 7

FIGURE 7. GPx and MnSOD overexpression increases cell death resistance to Cr(VI). *A*, Western blot analysis. BEAS-2B and H460 cells were transfected with GPx, MnSOD, or control pcDNA3 plasmid. β-actin was used as a loading control. *B and C*, Transfected cells were treated with 20 μM Cr(VI) for 12 h and the effect of GPx and MnSOD overexpression on apoptosis was determined by Hoechst 33342 assay. Plots are mean ± S.D. ($n = 3$). *, $p < 0.05$ versus mock-transfected Cr(VI)-treated controls.

Figure 8

FIGURE 8. Bcl-2 overexpression decreases Cr(VI)-induced apoptosis. *A*, Western blot analysis. BEAS-2B and H460 cells were stably transfected with myc-tagged Bcl-2 plasmid or control pcDNA3 plasmid. β -actin was used as a loading control. *B*, Transfected cells were treated with 20 and 50 μ M Cr(VI) for 12 h and apoptosis was determined by Hoechst 33342 assay. Plots are mean $\pm$ S.D. ($n = 3$). *, $p < 0.05$ versus mock-transfected controls.

Figure 9. **Prevailing model of carcinogenesis.** Normal epithelial cells (grey) become hyperproliferative (pink) following induction. As they reach the oxygen diffusion limit, they become hypoxic (blue), which can either lead to cell death (apoptotic cells shown with blebbing) or adaptation of a glycolytic phenotype (green), which allows cells to survive. As a consequence of glycolysis, lesions become acidotic, which selects for motile cells (yellow) that eventually breach the basement membrane.

Figure 10. **Mitochondrial ROS production and defense.** Superoxide ($O_2^{\bullet-}$) generated by the respiratory chain is mostly released to the matrix at complex I and the inter membrane space (IMS) at complex III. $O_2^{\bullet-}$ can naturally dismute to hydrogen peroxide (H_2O_2) or is enzymatically dismuted by matrix MnSOD (1) or Cu/ZnSOD (2) in the IMS or cytosol. H_2O_2 is detoxified in the matrix by catalase (3), the thioredoxin/thioredoxin peroxidase system (4), or the glutathione/glutathione peroxidase system (5). Alternately, H_2O_2 can react with metal ions to generate the highly reactive hydroxyl radical ($\cdot OH$) via Fenton chemistry (6). $O_2^{\bullet-}$ is not membrane permeable but can pass through ion channels (solid lines), whereas H_2O_2 can pass freely through membranes (dashed lines). Bcl-2 modulates MPTP opening as well as VDAC activity. $O_2^{\bullet-}$, superoxide; H_2O_2 , hydrogen peroxide; MnSOD, manganese superoxide dismutase; Cu/ZnSOD, copper/zinc superoxide dismutase; CAT, catalase; THD, NADH transhydrogenase; TR, thioredoxin reductase; TPx, thioredoxin peroxidase; TRx^{red} , reduced thioredoxin; TRx^{ox} , oxidized thioredoxin; GSH, glutathione; GSSG, glutathione disulfide; IMAC, inner membrane ion channel; VDAC, voltage dependant anion channel; MPTP, mitochondrial permeability transition pore; Bcl-2, B-cell lymphoma 2 antiapoptotic effector.

Figure 11. **Proposed mechanism contributing to Cr(VI)-induced toxicity and carcinogenesis**
 Cr(VI) accumulates in the mitochondrial matrix at higher relative concentrations than in the cytosol/nucleus and it significantly amplifies the ROS burden via **Fenton chemistry “explosion”** leading to acute and chronic (mDNA damage) **“respiratory defect”**. Consequently, mitochondrial signaling integrity is lost and the cell (1) amplifies glycolysis as the only remaining viable energy source and (2) loses commitment phase apoptotic signaling hub.

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CHAPTER III

Malignant Transformation and Tumorigenesis of Chromium (VI)- Exposed Lung Cells and their Inhibition by Bcl-2 Knockdown

3.1 Abstract

Environmental exposure to heavy metals such as hexavalent chromium (Cr(VI)) is associated with an increased incidence of human lung cancer. Elucidation of phenotypic changes, specific molecular effectors and their hierarchy in Cr(VI)-induced carcinogenesis has been limited due to the lack of appropriate experimental models especially for long-term exposure studies. We have developed an *in vitro-in vivo* chronic exposure model for mechanistic studies of Cr(VI)-induced carcinogenesis which is applicable to other xeno-carcinogenesis studies. Long-term exposure to Cr(VI) leads to malignant transformation of human lung epithelial BEAS-2B cells, as indicated by their increased cell migration, invasion, proliferation, and colony formation activities. Cr(VI)-transformed cells induced tumorigenesis in nude mice comparable to that of well established human lung cancer H460 cells, whereas passage-matched control BEAS-2B cells showed no tumor development. To provide a mechanistic insight to the tumorigenic process, stable knockdown mutants of Cr(VI)-transformed cells and H460 cells exhibiting downregulated Bcl-2 phenotype were generated and evaluated for tumor associated properties *in vitro* and *in vivo*. The Bcl-2 knockdown mutants showed a substantial decrease in the rates of colony formation, invasion, migration and proliferation, as well as tumor formation in mice, in comparison to vector controls. These results indicate a novel role of Bcl-2, beyond apoptosis regulation, to be a key regulator of malignant transformation and tumorigenesis *in vivo*. The model described here may provide utility to other xeno-carcinogenesis studies, including those of heavy metals which currently lack effective *in vivo* tumor development experimental means.

3.2 Introduction

Lung cancer is the leading cause of cancer mortality worldwide and yet the etiology of lung cancer is poorly understood. Current research indicates that long-term exposure to inhaled carcinogens has the greatest impact on the risk of lung cancer. Cr(VI) compounds are ubiquitous carcinogens associated with the incidence of lung cancer in humans. Several epidemiological studies in the last few decades have associated exposure to Cr(VI) with the induction of lung cancer in workers in various occupational settings (1-5). Cr(VI) compounds are also present in cigarette smoke, automobile emissions, and are widespread in the environment, e.g., Cr(VI)-contaminated water. In the United States, an air quality survey indicated that people in several residential areas are exposed to particulate airborne chromium at concentrations exceeding 100 times the chronic toxicity benchmark (6). Therefore, in addition to occupational exposure, environmental chromium is an emerging concern for its associated long-term carcinogenic effect of the lungs.

Although several epidemiological studies have demonstrated the carcinogenic effect of Cr(VI) compounds, animal studies have yielded negative or inconsistent results (7-9) due to genetic variations or other predisposing factors that are not clearly understood. The lack of appropriate animal models has hindered the efforts to identify the mechanisms of Cr(VI)-induced carcinogenesis; therefore, even though Cr(VI) compounds have been identified as human carcinogens (10), the underlying mechanisms remain poorly understood. To date, most Cr(VI) carcinogenesis studies have focused on short-term or acute effects; however, carcinogenesis is a multi-step process requiring long-term exposure to the carcinogen. To mimic the pathologic exposure condition, we developed an *in vitro* chronic exposure model combined with *in vivo* tumorigenesis model to aid mechanistic studies of Cr(VI)-induced lung carcinogenesis. Using the combined model, we also investigated the potential role of Bcl-2 in the carcinogenic process.

Bcl-2 is a key apoptosis-regulatory protein known to be important in the regulation of Cr(VI)-induced apoptosis (11). Chronic exposure of lung epithelial cells to Cr(VI) led to apoptosis resistance and upregulation of Bcl-2 (12). Several apoptosis-resistant lung cell lines and tumor specimens have also been shown to overexpress Bcl-2 (13-15). This protein has also been shown to be upregulated in many forms of cancer, including 90% of colorectal cancer, 70% of breast cancer, and 30-60% of prostate cancer (16, 17). While these studies suggest the possible role of Bcl-2 in Cr(VI)-induced carcinogenesis, direct evidence for its role is lacking. In this study, we used a molecular approach to knockdown Bcl-2 and measured its effects on malignant transformation and tumorigenic properties of human lung epithelial cells chronically exposed to Cr(VI). We also used bioinformatics approach to aid the mechanistic investigations of Bcl-2's role in Cr(VI)-induced carcinogenesis.

3.3 Materials and Methods

Chemicals and Reagents

Sodium dichromate ($\text{Na}_2\text{Cr}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$) [Cr(VI)] was obtained from Sigma (St. Louis, MO). Hoechst 33342 were obtained from Molecular Probes (Eugene, OR). Lipofectamine was from InVitrogen (Carlsbad, CA). Antibodies for Bcl-2, β -actin, and peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

Human lung epithelial BEAS-2B cells and human lung cancer epithelial H460 cells were obtained from American Type Culture Collection (Manassas, VA). BEAS-2B cells were cultured in Dulbecco's modified eagle medium containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin and streptomycin. H460 cells were cultured in RPMI 1640 medium containing 10%

FBS, 2 mM L-glutamine, 100 units/ml penicillin and streptomycin in a 5% CO₂ environment at 37°C.

Chronic Cr(VI) Exposure

BEAS-2B cells were continuously exposed to 5 µM Cr(VI) in culture in 5% CO₂ at 37°C. The cells were passaged weekly at preconfluent densities using a solution containing 0.05% trypsin and 0.5 mM EDTA. Cr(VI)-exposed BEAS-2B cells are designated as BEAS-Cr cells so as to distinguish them from the parental BEAS-2B cells. Parallel cultures grown in Cr(VI)-free medium provided passage-matched controls. After 24 weeks of exposure, Cr(VI)-exposed cells were cultured in normal medium and their tumorigenic potential was assessed by various measurements below.

Cell Growth Assay

Cells (1×10^5 cells) were seeded on 60-mm cell culture dishes and were incubated at 37°C in a 5% CO₂ incubator. At specific times after the incubation, cells were trypsinized and analyzed for cell number using Countess[®] automated cell counter (Invitrogen, Carlsbad, CA).

Soft Agar Colony Formation Assay

Soft agar assay was performed as previously described with minor modifications (18). The cell lines studied (3×10^4 cells) were mixed with tissue culture medium containing 0.5% agar to result in a final agar concentration of 0.33%. Cell suspension (1.5 ml) was plated in 60-mm dishes coated with 7 ml of 0.5% agar in tissue culture medium. After 2 weeks, the average number of colonies with a diameter of more than 50 cells was scored under a light microscope.

Invasion Assay

Invasion assays were performed using Transwell invasion chambers coated with Matrigel (50 µl per filter) (BD, USA) according to the manufacturer's protocol. Cells were cultured for 48 h and transferred on the top of Matrigel-coated

invasion chambers in a serum-free medium (1×10^5 cells per Transwell). Medium containing 5% FBS was added to the lower chambers. After incubation for 24 h, cells that remained on the top of the filter were scrubbed off and cells that migrated to the lower surface were fixed in 90% alcohol and followed by a crystal violet stain.

Migration Assay

Cell migration was determined by wound assay. Cells were cultured in 24-well plate and then a wound space was made with 1 mm width tip. Cells were incubated under standard conditions for 24 h, after which they were washed and examined under a light microscope. Analysis of cell migration was performed by using an average wound space from ten random fields of view, and the percentage of change in the wound space was calculated using the formula: % change = (average space at time 0 h) - (average space at time 24 h) / (average space at time 0 h) x 100. Relative cell migration was calculated by dividing the percentage change in the wound space of indicated cells by that of the control cells in each experiment.

Generation of Bcl-2 Knockdown Clones

Lentiviral transduction particles carrying short hairpin RNA (shRNA) sequence against human Bcl-2 (5'-CCGGGTGATGAAGTACATCCATTATCTCGAGATAATGGATGTA CTTTCATCACTTTTG-3') and control non-target sequence (5'-CCGGC AACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT-3') were used to knockdown Bcl-2 expression in BEAS-Cr and H460 cells. The viral vectors were obtained from Sigma (Cat # SHVRS-NM_000633 and SHC002V, respectively) and were used according to the manufacturer's protocol. Briefly, the cells were seeded in 6-well plates (5×10^5 /well) and incubated with Bcl-2 shRNA lentiviral particles or control particles at the multiplicity of infection (MOI) of 1.5 in the presence of hexadimethrine bromide (8 μ g/ml) for 36 h. For generation of stable clones, cells were plated onto 10-cm dishes and cultured for 14 d with puromycin (1 μ g/ml). Resistant clones expressing varying levels of Bcl-

2 were isolated using cloning cylinders (Bellco Glass, Vineland, NJ) and transferred for expansion and analysis by Western blotting.

Western Blotting

Cells were incubated in a lysis buffer for 20 min on ice. After centrifugation at 14,000g for 15 min at 4°C, cell supernatants were collected and assayed for protein content. Equal amounts of protein per sample (15 µg) were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto 0.45-µm nitrocellulose membranes. The transferred membranes were blocked for 1 h in 5% non-fat dry milk in TBST (25 mM Tris-HCl, pH 7.4, 125 mM NaCl, 0.05% Tween-20) and incubated with appropriate primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. The immune complexes were detected by chemiluminescence (Supersignal® West Pico, Pierce Biotechnology, Rockford, IL) and quantified by imaging densitometry using UN-SCAN-IT automated digitizing software (Silk Scientific, Orem, UT). Mean densitometry data from independent experiments were normalized to the control.

In Vivo Tumor Xenograft Model

Athymic (nu/nu) male nude mice, obtained from Jackson Laboratory (Bar Harbor, ME), were housed under pathogen-free conditions and fed autoclaved diet and water ad libitum. To establish tumor xenografts in mice, BEAS-2B, BEAS-Cr, H460 and Bcl-2 knockdown cells (1×10^6 cells) were suspended in 1:1 medium mixed with Matrigel and were injected subcutaneously on the left and right flanks of each mouse. Tumor size was measured at 14 d post-injection by using a caliper, and the tumor volume was determined using the formula: $0.5238 \times L$ (length) $\times W$ (width) $\times H$ (height) of the tumor. All procedures were conducted in accordance with the guidelines for the Use and Care of Laboratory Animals and approved by the Institutional animal care and use committee.

Bcl-2 Interactome Analysis

The Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems, Redwood City, CA) was used to analyze currently published Bcl-2 interactome. The Ingenuity database is the largest curated database of previously published findings on mammalian biology from the public literature (i.e., MEDLINE). Reports on individual studies of genes in human, mouse or rat were first identified from peer-reviewed publications, and findings were then encoded into ontology by content and modeling experts. Network analysis using the knowledge base was used to further identify direct interactions between mammalian orthologues. The Bcl-2-interactome is a graphical representation of its published molecular relationships. Molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). Nodes are displayed using various shapes that represent the functional class of the gene product. Edges are displayed with various labels that describe the nature of the relationship between the nodes.

Statistical Analysis

The data were expressed as means $\pm$ SD of three or more independent experiments. Statistical analysis was performed using two-tailed and paired Student's t-test. P values less than 0.05 were considered statistically significant and indicated by an asterisk.

3.4 Results

Cell Growth and Colony Formation

BEAS-2B cells were continuously exposed to Cr(VI) for 24 weeks in culture as described in *Materials and Methods*. In order to assess the relative tumorigenic potential of the exposed cells (BEAS-Cr), we compared their growth and colony formation characteristics to those of the passage-matched BEAS-2B cells serving as a negative control, and human lung carcinoma H460 cells serving as a positive control. The BEAS-2B cell line has been used previously to study the carcinogenic potential or effects of various chemicals and biological agents (19-

21). The H460 cell line has been evaluated extensively in mechanistic studies of human lung cancer. Statistically significant increase in growth capacities was observed as early as 48 h in BEAS-Cr and H460 cells as compared to control BEAS-2B cells (Figures 1A-B). By 96 h, the growth rates of BEAS-Cr and H460 cells were more than double that of the control BEAS-2B cells. Next, soft agar colony formation assay was performed to assess relative colony formation capacities. BEAS-2B, BEAS-Cr, and H460 cells were subjected to colony formation assay by growing them on agar plates so as to assess anchorage-independent growth. After 2 weeks, significant colony formation was observed in BEAS-Cr cells with a 7-fold increase as compared to passage-control BEAS-2B cells which also formed very few, slow growing colonies (Figures 1C-D). The H460 human lung cancer cells exhibited the highest colony formation capacity, out-growing the BEAS-2B cells by approximately 8-fold and BEAS-Cr cells by approximately 1.5-fold.

Migration and Invasion

We next investigated the relative, aggressive-malignant phenotype, capacities using the same comparative approach. The H460 cells showed the highest invasion capacity with BEAS-Cr cells invading at the rate of 70% relative to H460, and BEAS-2B invading at 15% (Figures 2A-B). A 7-fold increase in invasion rate was observed in BEAS-Cr as compared to BEAS-2B cells. The highest migration capacity was observed in H460 cells followed by BEAS-Cr cells, at approximately 75% of H460 cells, and finally, BEAS-2B cells, at less than 10% of H460 cells (Figures 2C-D). Together, these studies indicate that chronic exposure of non-tumorigenic BEAS-2B cells induced malignant transformation of the cells showing phenotypic characteristics similar to those of the tumorigenic H460 cells.

Bcl-2 Expression and Sensitivity to Cr(VI)

Apoptosis is the primary mode of cell death following an insult with Cr(VI) at physiologically relevant doses (11, 22). Apoptosis resistance, on the other hand,

is the central objective of “one renegade cell” on its quest toward malignancy. Previous studies have shown that Bcl-2 is a key protein involved in apoptosis resistance to Cr(VI) (11, 12). Whether this is a mechanistic feature unique to specific cell systems or a more general feature of carcinogenesis is uncertain. To test this possibility, we evaluated endogenous Bcl-2 protein expression across our 3 cell type comparative system using Western blots. The results show that BEAS-Cr cells exhibited a substantial higher amount of Bcl-2 relative to that of passage-control BEAS-2B cells, but comparable to that of H460 cells (Figure 3A). In response to acute Cr(VI) treatment, Bcl-2 protein expression was substantially downregulated in BEAS-2B cells, and to a much lesser extent in BEAS-Cr and H460 cells (Figure 3B), suggesting a mechanistic similarity between the latter two cell types.

Bcl-2 Knockdown Effect in Vitro

Mutant cell lines exhibiting stably downregulated Bcl-2 were generated using RNA interference and clonal selection. Maximum Bcl-2 downregulation in BEAS-Cr mutants (85%) was achieved in clone 2, which was used for the remainder of the study (Figure 4A). H460 clone 1 showed 75% Bcl-2 reduction and was used for further study. Scramble sequences were used to generate appropriate controls for the BEAS-Cr and H460 mutant cells. Downregulation of Bcl-2 reduced proliferation rates of both BEAS-Cr and H460 cells by about 50% at 96 h (Figure 4B). Next, the effect of Bcl-2 knockdown on colony formation capacity was evaluated. The BEAS-Cr mutant exhibited approximately 60% reduction in colony formation while a 40% reduction was observed in the H460 mutant as compared to their respective controls (Figure 4C). The trend continued when migration and invasion capacities were assessed with both mutants showing significant decrease in these tumor-associated properties compared to controls (Figures 4D-E).

Bcl-2 Knockdown Effect In Vivo

To assess the potential tumorigenicity of Cr(VI)-transformed BEAS-Cr cells and role of Bcl-2 in the tumorigenic process, experiments were performed using a xenograft mouse model. The cells and their knockdown mutants were subcutaneously injected into nude mice. At one week after the injection, small tumors were formed at the injection site in mice receiving BEAS-Cr and H460 cells, whereas mice receiving BEAS-2B cells did not develop tumors. At 14 days post-injection, large tumors were found in the BEAS-Cr (Figure 5A) and H460 (Figure 5B) mice, whereas no or very small lumps were observed in the BEAS-2B control mice. To our knowledge, this is the first demonstration of *in vivo* tumorigenesis with Cr(VI)-transformed cells. Mice receiving Bcl-2 knockdown mutants, either BEAS-Cr or H460, showed a substantial reduction in tumor volume as compared to their respective controls (Figures 5A-B). These results indicate the role of Bcl-2 in tumorigenesis of Cr(VI)-transformed cells. The inhibition of tumor growth in H460 mutant mice also implicates the general role of Bcl-2 in tumorigenesis of lung cancer cells.

Bcl-2 Interactome Analysis

In order to establish a strategy towards a better understanding of the mechanism(s) involved in Bcl-2's contribution to carcinogenesis, we performed an extensive PubMed database search for possible molecular targets. The initial query for Bcl-2 returned 33,970 hits! We realized that other approaches may need to be evaluated given the complexity of Bcl-2 cellular role(s). Following the analysis of available options, we turned to Ingenuity Pathways Analysis (IPA). IPA integrates the primary literature into easily searched and visualized networks allowing for orders of magnitude faster evaluation of cellular signaling cascades. Figure 6A shows the initial IPA query of Bcl-2's interactions reported to date (IPA v8.6). 741 molecules have thus far been reported to interact with Bcl-2 either directly or indirectly. In order to make the network more relevant to our model, we filtered for only those interactions reported in humans (Figure 6B). This reduced the interaction network to 56 molecules organized by cellular localization (Bcl-2-interactome). Given that we selectively targeted Bcl-2 and observed

significant consequences on tumor-associated properties, we asked IPA to map only the downstream components of human Bcl-2-interactome (Figure 6C). The query returned 20 molecules. As we progressed from Figure 6A overwhelming complexity to Figure 6C, the current mechanistic evidence of Bcl-2's role in carcinogenesis emerged, as did a new approach to scientific query.

3.5 Discussion

Although Cr(VI) has been identified as a potent human carcinogen, the mechanism underlying the carcinogenic process remains unclear due in part to the lack of appropriate experimental models for long-term exposure studies. In this study, we reported a combined *in vitro-in vivo* model for Cr(VI) carcinogenesis studies using chronically exposed bronchial epithelial BEAS-2B cells and a mouse tumorigenesis model. Bronchial epithelial cells were chosen in this study because they are a key target for Cr(VI)-induced carcinogenesis. BEAS-2B cells have been widely used in the literature to define conditions under which various agents and oncogenes cause neoplastic transformation (19-21). Inclusion of the passage-control BEAS-2B cells and bronchial carcinoma H460 cells into direct comparisons was critical when assessing the significance of tumorigenic properties in the evolved BEAS-Cr phenotype. H460 cells are a well established model for *in vitro* and *in vivo* mechanistic studies of lung carcinogenesis and therefore represent a positive control. For BEAS-Cr cells, significant growth increase was observed as early as 48 h and at 96 h the growth rate was double that of control BEAS-2B cells (Figures 1A-B). H460 cells proliferated even faster, at approximately 2.5 times that of BEAS-2B cells. This could be in part due to 10% serum content of standard culture medium for H460 cells vs. 5% for BEAS-2B and BEAS-Cr cells. We performed a series of direct comparisons with all cell lines cultured at 5% serum and observed a slight decrease in H460 cell growth (data not shown), however we opted to continue at 10% since it is the literature standard.

Anchorage-dependent growth is a typical feature of normal cells and controls cell division (23), however when cells are transformed they lose this property. Anchorage-independent growth has been widely correlated with tumorigenicity and invasiveness in several cancer cell types (23). Soft agar assay is a stringent test to study the ability of cells to undergo anchorage-independent growth. The number of colonies formed by BEAS-Cr cells was about 7 times more than that in control BEAS-2B cells, confirming the carcinogenic potential of Cr(VI)-transformed cells (Figures 1C-D). H460 cells formed colonies at even higher rate, exceeding BEAS-Cr cells by about 30%. Interestingly, bronchial epithelial BEAS-2B cells obtained at autopsy of non-cancerous individuals also formed a small number of slow growing colonies on soft agar. This could be due to the fact that BEAS-2B cells are reported to possess mutated and dysfunctional p53 tumor suppressor gene (19, 24). Additionally, the American Type Culture Collection (ATCC) indicates that the BEAS-2B cell line forms colonies in semisolid medium but is non-tumorigenic in immunosuppressed mice. Therefore, the colony formation observed in BEAS-2B cells is due to the indigenous properties of the cell and not because of their tumorigenic potential. Cr(VI)-transformed cells were further characterized for their invasive and migratory properties and compared to those of established lung cancer H460 cells. Invasion and migration were shown to increase significantly as compared to passage-control BEAS-2B cells (Figures 2A-B). Recent reports indicate that BEAS-2B are non-tumorigenic, aneuploid cells but can undergo squamous differentiation in response to serum and transforming growth factor- β (25). However, since passage-control BEAS-2B cells showed no phenotypic changes or malignant behavior, it can be concluded that BEAS-Cr cells are Cr(VI)-transformed BEAS-2B cells and not BEAS-2B cells that have undergone differentiation and show altered phenotype due to continued passaging.

Carcinogenesis is a multistep process involving progressive transformation of normal cells into malignant phenotypes along with the aberrant accumulation of such abnormal cells (26, 27). Cr(VI) may affect the carcinogenic process during

malignant transformation, i.e., by causing DNA mutation and damage, or aberrant cell accumulation, i.e., through evasion of apoptosis. Sensitivity to apoptosis depends on the expression level of various apoptosis-regulatory proteins. A functional loss of pro-apoptotic proteins and/or increased expression of anti-apoptotic proteins can confer resistance to apoptotic stimuli (28, 29). Bcl-2 is a key anti-apoptotic protein involved in the apoptosis resistance to Cr(VI) (11). A lesser known function of Bcl-2 is its tumorigenic properties. Bcl-2 expression has been reported to be elevated in many forms of cancer (13-17) and was found in this study to be upregulated in Cr(VI)-transformed BEAS-Cr cells (Figure 3A). In order to evaluate the potential relationship between Bcl-2 and tumorigenic properties, gene knockdown experiments were performed to allow direct assessment of the Bcl-2's contribution to tumorigenic phenotype of BEAS-Cr and H460 cells. Our results show that Bcl-2 knockdown leads to significant reduction of all tumorigenic properties evaluated (Figures 4A-E). While Bcl-2 is a well established oncogene, its 'oncogene' status is largely attributed to the evolution of chemoresistance via modulation of cell's apoptotic machinery. To the best of our knowledge, this is the first demonstration of Bcl-2's role in carcinogenesis well beyond apoptosis regulation to that of "master" oncogene. Our *in vivo* data (Figures 5A-B) confirm this assertion. We show that Cr(VI)-transformed BEAS-Cr cells form large tumors in nude mice, whereas control BEAS-2B cells do not. This is the first report of Cr(VI)-transformed cells exhibiting such *in vivo* properties. Furthermore, downregulation of Bcl-2 led to a substantial reduction of tumor volume in BEAS-Cr derived tumors. This mechanistic feature was nearly identical in human lung cancer H460 cells' derived tumors, indicating a potential shared mechanism between laboratory Cr(VI)-induced carcinogenesis and human lung cancer.

Finally, Ingenuity Pathways Analysis revealed the known Bcl-2-interactome containing 56 molecules and 56 relationships identified as of IPA v8.6 (Figure 6B). Given that we were interested in mechanisms underlying the effects observed following the downregulation of Bcl-2, we chose to focus on Ingenuity's

output of known downstream effectors of Bcl-2-interactome (Figure 6C). Did any of the molecules listed have a link to regulation of tumor-associated properties such as proliferation, transformation, migration, invasion and colony formation? Analysis of Ingenuity Knowledge Base (IKB) strongly suggests YES. Out of all downstream Bcl-2 effectors, nucleus-localized constituents strongly argue that Bcl-2's role in carcinogenesis exceeds that of apoptosis regulation in line with our observations. Cyclin-dependent kinases 1 and 2 (CDK1&2) regulate cell cycle progression, cellular motility and proliferation according to IKB summary of over 6,000 categorized literature findings. Their role in carcinogenesis is well established. Next, telomerase (TERT) is an established positive regulator of cell proliferation, immortalization and transformation. Its role in carcinogenesis is widely accepted. Of interest, RAD9A, implicated in the regulation of colony formation also appears in the IPA's output. Relative to its "neighbors", it is a less studied protein with only 380 categorized literature findings in the IKB. Lastly, vascular endothelial growth factor (VEGF) is a well established positive regulator of angiogenesis and is a downstream target of Bcl-2. Its role in Cr(VI)-induced carcinogenesis has not been demonstrated but is implicated by our study. In conclusion, evidence presented in this report suggests Bcl-2's role in carcinogenesis is much more than apoptosis evasion to that of apoptosis-proliferation network hub regulator. Animal and *in vitro* data support its role in Cr(VI)-induced tumorigenesis and malignant transformation. The reported experimental models may be used to aid mechanistic studies of other carcinogens.

Figure 1

FIGURE 1. Cell growth and colony formation capacities of BEAS-2B, BEAS-Cr and H460 cells. A, BEAS-2B, BEAS-Cr and H460 cells (1×10^5 cells) were seeded on 60-mm cell culture dishes and were incubated at 37°C in a 5% CO_2 incubator. At the indicated times, cells were trypsinized and analyzed for cell number using an electronic cell counter. B, Representative light micrographs of BEAS-2B, BEAS-Cr and H460 cells in culture at 96 h are shown. C, BEAS-2B, BEAS-Cr and H460 cells (3×10^4 cells) were seeded on 0.5% agar plates and were incubated at 37°C in a 5% CO_2 incubator. After 2 weeks, colony formation capacity was scored under a light microscope. D, Representative micrographs of colonies formed by BEAS-2B, BEAS-Cr and H460 cells on soft agar are shown. Values are mean $\pm$ S.D. ($n = 4$). * $p < 0.05$ versus passage-control BEAS-2B cells.

Figure 2

FIGURE 2. *In vitro* relative malignant phenotype capacities of BEAS-2B, BEAS-Cr and H460 cells. **A**, BEAS-2B, BEAS-Cr and H460 cells (1×10^5 cells) were added to Transwell inserts coated with Matrigel and incubated for 24 h. Invading cells were stained and counted under a light microscope. Plots show relative invasion of BEAS-2B, BEAS-Cr and H460 cells. **B**, Representative micrographs of cells stained for invasion are shown. **C**, Confluent monolayers of BEAS-2B, BEAS-Cr and H460 cells were wounded, and the cells were allowed to migrate for 24 h. Wound space was visualized under a phase-contrast microscope and analyzed by comparing the relative change in wound space as compared to control cell monolayers. **D**, Representative micrographs of cells migrating from the wound edge are shown. Values are mean $\pm$ S.D. ($n = 4$). * $p < 0.05$ versus passage-control BEAS-2B cells.

Figure 3

FIGURE 3. Bcl-2 expression and response to Cr(VI) treatment in BEAS-2B, BEAS-Cr and H460 cells. *A*, Endogenous Bcl-2 levels in BEAS-2B, BEAS-Cr and H460 cells determined by Western blotting. Densitometry was performed to determine the relative Bcl-2 levels after reprobing the membrane with β -actin antibody. *B*, Cells were either left untreated or treated with Cr(VI) (10, 20 μ M) for 12 h. Cell lysates were prepared and analyzed for Bcl-2 by Western blotting. Values are mean $\pm$ S.D. ($n = 4$). * $p < 0.05$ versus passage-control BEAS-2B cells; # $p < 0.05$ versus non-treated control of the respective cells.

Figure 4

FIGURE 4. Effect of Bcl-2 knockdown on tumor-associated properties *in vitro*. **A**, Bcl-2 knockdown experiments were performed in BEAS-Cr and H460 cells by infecting the cells with Bcl-2 shRNA (shBcl-2) viral particles or control shRNA (shCon) particles as described in "Materials and Methods". Clonal selection and Bcl-2 expression were performed to identify mutants with downregulated Bcl-2. **B**, Effect of Bcl-2 on *in vitro* cell growth of the respective cell lines and their mutants. Cells (1×10^5 cells) were seeded and incubated at 37°C for 96 h, after which they were analyzed for cell number. **C**, Cells (3×10^4 cells) were seeded on soft agar plates and the colonies were scored after 2 weeks. **D**, Invasion assays were performed using Transwell invasion chambers as described. **E**, Cell migration was determined by wound assay as described. Values are mean $\pm$ S.D. ($n = 3$). * $p < 0.05$ versus passage-control BEAS-2B cells or shCon cells.

Figure 5

FIGURE 5. Effect of Bcl-2 knockdown on tumor-associated properties *in vivo*. A, Mice were injected subcutaneously with 1×10^6 passage-control BEAS-2B, BEAS-Cr, or shBcl-2 BEAS-Cr mutant cells. Tumor formation was determined at 14 d post-injection. Representative photographs are shown. B, Mice were similarly injected with control BEAS-2B, H460, or shBcl-2 H460 cells. Tumor formation and representative micrographs at 14 d post-injection are shown. Data are means $\pm$ S.D. ($n = 4$). * $p < 0.05$ vs. passage-control BEAS-2B cells.

Figure 6A

Figure 6B

Figure 6C

FIGURE 6. Ingenuity Pathways Analysis software output for the Bcl-2 interactome. A, Initial IPA query for the Bcl-2-interactome. 741 molecules were reported (as of IPA v8.6, October 5, 2010). B, Bcl-2-interactome filtered for interactions reported in humans only and presented by cellular localization. 56 molecules were reported to interact either directly or indirectly. C, Downstream components of human Bcl-2-interactome. 20 molecules were reported to date.

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OVERALL CONCLUSIONS

- Cr(VI) induces apoptosis in cancerous H460 cells and non-cancerous BEAS-2B cells in a dose dependent manner.
- Cancerous H460 cells exhibit 4-fold native resistance to Cr(VI)-induced apoptosis.
- The native apoptosis resistance of cancerous H460 cells is abrogated when oxidative phosphorylation-only metabolism is forced.
- ROS play a key role in Cr(VI)-induced apoptosis signaling.
- The primary, apoptosis relevant, source of ROS is mitochondrion.
- mROS intensity is increased in cancerous H460 cells but not in non-cancerous BEAS-2B cells when oxidative phosphorylation-only metabolism is forced.
- Inhibition of mROS as well as outer mitochondrial membrane signaling (via Bcl-2) leads to inhibition of Cr(VI)-induced apoptosis.
- Prolonged exposure of non-cancerous BEAS-2B cells to sub-lethal Cr(VI) dose leads to transformation and evolution of malignant phenotype.
- Cell growth, colony formation, migration and invasion capacities of transformed BEAS-2B (BEAS-Cr) cells are substantially elevated *in vitro*.
- Stable and selective knockdown of Bcl-2 leads to significant reduction in tumor-associated properties *in vitro* in human lung cancer H460 and laboratory evolved malignant BEAS-Cr cells.
- Stable and selective knockdown of Bcl-2 leads to comparable reduction in tumor-associated properties *in vivo* in human lung cancer H460 and laboratory evolved malignant BEAS-Cr cells.
- Cr(VI)-induced malignant transformation of human lung epithelial cells is of clear relevance to human exposure and validates human lung epithelial cells as a direct target for Cr(VI)-induced carcinogenesis.

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OBJECTIVE

Continue the quest for deeper understanding of mitochondrial molecular biology and physiology.

EDUCATION

- **PhD**, Pharmaceutical and Pharmacological Sciences, May 2011, Department of Basic Pharmaceutical Sciences, West Virginia University, WV, USA.
- **MS**, Pharmaceutical Sciences, May 2003, Department of Basic Pharmaceutical Sciences, West Virginia University, WV, USA.
- **BS**, Biology with Minor in Chemistry, May 1998, West Virginia Wesleyan College, Buckhannon, WV, USA

MERITS & HONORS

Research Awards:

- Research Award in Pharmaceutical Biotechnology - American Association of Pharmaceutical Scientists (sponsored by Pfizer Global Biologics) (2004).

SKILL SET

- Development and validation of small-molecule bioanalytical methods (U/HPLC-MS/MS, Fluorescence, UV) in high-throughput, robotics supported environment meeting FDA GLP guidelines for human clinical studies.
- Design, evaluation and integration of new technological platforms for advancement of analytical capabilities

- Design of FDA compliant bioanalytical laboratory standard operating procedures
- Cell culture of various human cell lines.
- Assays for cell proliferation, migration, invasion, viability and death.
- Western blotting, immunoprecipitation, fluorescence microscopy.
- DNA and siRNA transfection, preparation of stable cell-lines.
- DNA cloning using PCR and Site-directed mutagenesis.
- Flow cytometry, spectrophotometry, spectrofluorometry.
- ELISA, soft agar assay
- DNA fragmentation assays

PUBLICATIONS.

SCIENTIFIC PAPERS:

- **Medan D**, Wang L, Lu Y, Davis ME, Jiang BH, Barnett JB, Guo L, Rojanasakul Y. Malignant transformation and tumorigenesis of chromium (VI)-transformed lung cells and their inhibition by Bcl-2 knockdown. Submitted.
- **Medan D**, Wang L, Lu Y, Rojanasakul Y. Energy substrate modulates Cr(VI)-induced apoptosis in human lung cancer cells via a mitochondrial reactive oxygen species-dependent mechanism. Submission preparation.
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WORK EXPERIENCE

- **June 1998-March 2008** – Bioanalytical Laboratory, R&D, Mylan Pharmaceuticals Inc.
Titles held:
Chemist 1998-2000
Senior Chemist 2000-2003
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- **WVU School of Pharmacy**, Concurrent with Mylan 2000-2008, 2008-Present
- **Mentor – Dr. Yon Rojanasakul**
 - Identification of key molecular targets involved in lung cancer induced by environmental and occupational carcinogens such as hexavalent chromium [Cr(VI)] compounds.
 - Findings will recognize novel therapeutic targets and important biological mediators such as reactive oxygen species in lung carcinogenesis induced by Cr(VI) and other mutagenic agents.

Mentored two graduate students and 4 summer interns during the period of my graduate work at WVU. Currently, one graduate student is an assistant professor and the other one is pursuing a graduate career.

- **Aug 2008 - June 2010 Teaching Assistant**, Department of Basic Pharmaceutical Sciences, West Virginia University.
 - Taught and managed pharmacy, graded homework, proctored exams, conducted and supervised experiments for PCL-1 (Pharmacy) labs.

PROFESSIONAL AFFILIATIONS

- Student member of the American Association of Pharmaceutical Scientists (AAPS) since 2001.
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INTERESTS

- Information technology
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- Real Estate
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