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Mechanisms of environmental carcinogenesis and metal -induced cellular signaling

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Mechanisms of environmental carcinogenesis and metal-induced cellular signaling.

Jacquelyn Jo Bower

**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 Sciences**

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2006

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ABSTRACT

Mechanisms of environmental carcinogenesis and metal-induced cellular signaling.

Jacquelyn Jo Bower

Low dose chronic exposure to environmental carcinogens is a major cause of human cancers. More than 375 known or suspected environmental carcinogens have been identified, many of which are transition metals or metal containing compounds (IARC, updated to 2004, <http://www-cie.iarc.fr/monoeval/crthall.html>). Although much research has focused on the ability of these metals to induce reactive oxygen species formation (ROS), DNA damage, and apoptosis, less effort has been put forth to examine the cellular signaling mechanisms responsible for these effects. Our experimental research has focused on the signaling pathways induced in response to arsenic exposure. Arsenic is a highly interesting transition metal due to its widespread exposure and paradoxical ability to induce carcinogenesis as well as apoptosis. Here, we highlight the importance of the human genome project in advancing the knowledge of the molecular mechanisms of metal-induced toxicology/carcinogenesis. In addition, we review the latest research in the field of metal-induced carcinogenesis. Finally, we describe a mechanism for the induction of the growth-arrest and DNA damage inducible protein 45 alpha (GADD45 α) involving arsenic-induced ROS formation in the non-tumorigenic human lung airway epithelial cell line, BEAS-2B.

Dedicated to
My Family,
Dennis and JoAnn Bower,
Elizabeth Hooks, Kathryn Bower,
Matthew Hirsch, and Neo the Wonderdog.

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SECTION I: OVERVIEW

Arsenic

Arsenic is a ubiquitous environmental contaminant found in food, water, cigarette smoke, soil, pesticides, and wood preservatives; thus, exposure can occur through dermal contact, inhalation, ingestion, and occupational routes (1-8). A multitude of adverse health effects, including hyperkeratosis, hyperpigmentation, diabetes mellitus, cardiovascular disease, reproductive defects, and cancers of the liver, bladder, skin, lung, kidney, and prostate, have been associated with arsenic exposure (9-17). The most common route of exposure is the ingestion of arsenic contaminated drinking water, which has been documented in Latin America, South Asia, Taiwan, the United States, and Japan at concentrations as high as 1.5-3.4 mg/L (18-25). These figures exceed the World Health Organization's maximum contaminant level of 10 ug/L by ten-fold or more. In the U.S. alone, an estimated 56 million Americans are exposed to concentrations of arsenic known to cause cancer (18,19,23-25).

Arsenic was first suggested to act as a human carcinogen by Hutchinson in the year 1887 when he noticed an abnormal number of skin cancers occurring in patients who were being treated with arsenicals (19). Since then, epidemiological studies have shown that arsenic exposure is associated with cancers of the skin, lung, liver, bladder, and prostate (9-12). In 1980, Rossman *et al* showed that inorganic arsenic was poorly mutagenic and genotoxic in both *E. coli* and CHO cells (26). Although arsenic induces DNA adduct formation, DNA-protein crosslinks, and ROS formation in vitro, attempts to examine the *in vivo* carcinogenic effects of arsenic in rodent models have largely failed in the absence of another carcinogenic agent (27-29).

Because of arsenic's poor mutagenicity, the current theory is that its carcinogenic effects are mediated by epigenetic mechanisms. This hypothesis is supported by the studies of Chen *et al*, in which overexpression of the oncogene c-myc and DNA hypomethylation of the promoter region of the estrogen receptor alpha gene have been observed in liver tumors of adult rats exposed to arsenic *in utero* (30,31). Additional supporting evidence includes the identification of the Erk MAPK as an essential component of the signal transduction pathways responsible for arsenic-induced transformation of JB6 murine fibroblast cells *in vitro* (32). Other studies have suggested that arsenic may prevent signaling events leading to cellular differentiation. For example, inorganic arsenic has also been shown to suppress involucrin expression, a marker of keratinocyte differentiation (33).

Although the previously described studies strongly suggest that gene expression and protein signaling events activated and suppressed during arsenic exposure play a central role in arsenic-induced tumorigenesis *in vitro*, knowledge of the signaling mechanisms responsible for transformation in a human model system remain virtually non-existent. Several colleagues have examined the transcriptional profiles of cell lines and tissues that have been exposed to arsenic. Examples include Liu's group (34), who showed that hepatocellular carcinoma induced by arsenic exposure *in utero* upregulated the expression of 56 genes and downregulated the expression of 26 genes when compared to non-tumor liver samples from CH3 mice. Moreover, gene expression changes in a BEAS-2B cell line differed between low (5 μ M) and high (50 μ M) doses of arsenic. In the high dose samples, general stress response genes were induced, whereas more subtle changes were observed at a lower dose of arsenic (35).

To date, only three groups have examined the effects of arsenic in mammalian models on a proteomic scale (36-38). Yet, both Lau's and He's groups used a rat lung epithelial cell line in which arsenic-induced transformation was not observed (37). In addition, He's group used the known carcinogen benzo-a-pyrene in conjunction with arsenic treatment (36). Furthermore, Wang's group chose to study only the nuclear matrix fraction of the K562 chronic myelogenous leukemic cell line in which arsenic is known to induce apoptosis, not transformation (38). In spite of the valuable information provided by these studies, an understanding of the signaling pathways responsible for arsenic-induced carcinogenesis is still in its infancy.

GADD45 α

One epigenetic mechanism by which arsenic may induce carcinogenesis is through the dysregulation of the cell cycle. Previous research from our laboratory has suggested that arsenic can increase the number of BEAS-2B cells found in the G₂/M phase fraction in a dose-dependent manner (39). The GADD45 α protein is a major player in the G₂/M phase checkpoint, and a transcriptional profile analysis of the BEAS-2B cells showed that the *gadd45a* gene is upregulated in response to both low and high dose exposure to arsenic (35). We therefore hypothesized that GADD45 α may be involved in arsenic's ability to induce G₂/M phase arrest.

The involvement of GADD45 α in the G₂/M phase checkpoint is thought to occur through its interaction with the cyclin dependent kinase cdc2 (also known as cdk1). GADD45 α is thought to bind to cdc2 and sequester it in the cytoplasm preventing cdc2 from associating with its cyclin counterpart, cyclin B1 (40). Recombinant GADD45 α

(rGADD45 α) protein expression has been shown to prevent cdc2 from immunoprecipitating with cyclin B1, thus specifically inhibiting cyclin B1/cdc2-dependent phosphorylation of its target protein histone H1 *in vitro* (40).

Additional functions of GADD45 α have also been proposed. For example, Smith *et al* have shown that GADD45 α and the proliferating cell nuclear antigen (PCNA) coimmunoprecipitate in ML-1 human myeloid leukemia cells containing a functional p53 gene upon exposure to ionizing radiation (IR) (41). PCNA is a sliding clamp ring protein that functions in DNA synthesis and recruits DNA repair proteins to the site of DNA damage (42). Furthermore, rGADD45 α addition has also been shown to stimulate nucleotide excision repair (NER) in both ML-1 and RKO cells (41). Taken together, these results suggest that PCNA may recruit GADD45 α to the site of DNA damage during cellular proliferation, implying a role for GADD45 α as a link between the DNA damage sensing machinery and DNA damage repair.

GADD45 α has also been shown to play a role in G₁/S phase arrest. Exposure of mouse embryo fibroblasts to IR can induce a p53-dependent G₁/S phase arrest (43). GADD45 α mRNA was increased after IR in the wild-type p53 ML-1, RKO, WI38, 344, U2-OS cell lines, but not in p53-deficient cell lines (43). Furthermore, p53 deficiency prevented GADD45 α mRNA induction and led to a loss of G₁ arrest after IR (43). An alternative mechanism of GADD45 α action at the G₁/S phase checkpoint suggests that it can prevent DNA synthesis from occurring, since a Balb/c-3T3 fibroblast GADD45 α -IPTG-inducible cell line can inhibit entry into S phase when IPTG is present (44).

Another function of the GADD45 α protein is to allow for topoisomerase I relaxing and cleavage activity (45). Addition of GADD45 α and core histones can relax

supercoiled DNA (45). GADD45 α can directly bind histones H1, H2A, H2B, H3, and H4, and subsequently interfere with their ability to interact with DNA (45). Furthermore, DNase I sensitivity increased when GADD45 α was added in the presence of hyperacetylated nucleosomes (45). GADD45 α can bind only hyperacetylated nucleosomes (not underacetylated) and can also bind UV-damaged DNA mononucleosomes and free DNA damaged by UV (45). The region of the protein responsible for these interactions is contained in amino acids 71-124 (45). Interestingly, this is the same region of the GADD45 α protein responsible for preventing cdc2/cyclin B1 kinase activity and phosphorylation of histone H1.

Regulation of GADD45 α

gadd45a is an inducible gene that was isolated as a novel mRNA increased after DNA damage (46). It is induced by many different types of cellular stress including methylmethane sulfonate (MMS) treatment, medium starvation, ionizing radiation (IR), ultraviolet radiation (UV), and sodium arsenite (47). Depending on which type of stressor is present in the system, the mechanisms of *gadd45a* induction differ. The most studied mechanism is the p53-dependent mechanism; however, a p53-independent mechanism also exists.

In some cases, the first step required for the transcription of inducible genes is the remodeling of nucleosomes. This allows the transcription factor to bind to the inducible gene and increase gene expression. In other cases, the nucleosomes have already been arranged around the transcription factor binding sites, allowing for quick binding of the selected transcription factor at any time. The *gadd45a* gene is thought to be one of these

so-called preset genes, in which the nucleosomes are arranged such that the promoter region and the 3rd intron are poised for easy access to transcription factors, since these regions are hypersensitive to DNase cleavage (48). Therefore, most studies of *gadd45a* transcriptional regulation have focused on the promoter and 3rd intron regions.

p53

The p53-dependent mechanism of *gadd45a* induction is usually seen in response to IR exposure. Graunke *et al*, have shown that IR treatment causes a 15-fold induction of *gadd45a* mRNA as measured by northern blotting in the absence of *de novo* protein synthesis (48). In addition, Zhan *et al* have shown that the p53-dependent induction of *gadd45a* occurs via p53 binding to its consensus sequence in the 3rd intron of the *gadd45a* gene and subsequently interacting with the WT1 transcription factor (49). However, this response seems to be strictly employed only with exposure to IR.

Oct-1

Oct-1 is a ubiquitously expressed cis-regulatory transcription factor of the POU (*pit-oct-unc*) family. It has been shown to regulate the transcription of many genes, including histone H2B, the small nuclear RNA gene, immunoglobulin genes in B cells, the von Willebrand factor, TIF2, GnRH, and the vascular cell adhesion molecule (VCAM) gene (50,51). The Oct-1 transcription factor has also been shown to bind the promoter region of the *gadd45a* gene between -101 to -82 bp relative to the transcriptional start site (TSS) in a p53-independent manner following MMS treatment and UV irradiation (50-53).

Takahashi and colleagues have suggested that a cofactor is needed in order for Oct-1 to induce *gadd45a* transcription in response to UV treatment (50). Jin *et al* have suggested that this cofactor may be the ubiquitous NF-YA transcription factor which binds to a CAAT-box motif located in the same region of the *gadd45a* promoter as the Oct-1 binding motif. Both Jin and Zhao found that Oct-1 is induced in response to MMS treatment, which contradicts Takahashi's results suggesting that Oct-1 abundance was not increased in response to UV stimulus (50-52). Jin also suggests that Oct-1 protein induction is mediated through a post-transcriptional mechanism (PTM), whereas Takahashi claims that the Oct-1 protein is not modified (50,51). These differences could be due to differences in the cell lines examined or the stimulus used for the induction of *gadd45a*. Both Takahashi's and Jin's work is supported by that of Graunke *et al*, in which LMPCR was used to identify potential protein binding interactions, one of which was the Oct-1 transcription factor (48).

ZBRK1

ZBRK1 is a zinc finger protein that is thought to repress *gadd45a* transcription by binding to the 3rd intron in a BRCA1-dependent manner (54). BRCA1 itself cannot bind directly to a regulatory DNA sequence; it must associate with a transcription factor that is capable of binding to a specific DNA sequence. There may be additional ZBRK1 binding sites in the *gadd45a* promoter region (54). Many other genes besides *gadd45a* contain putative ZBRK1 binding sequences, including GADD153, Ki-67, Bax, p21, EGR1, amphiregulin, prothymosin, TIMP-1, TIMP-2, and Topo IIa, suggesting that ZBRK1 may be involved in the cellular response to DNA damage (55). Yun *et al* have

recently shown that upon UV and MMS exposure, ZBRK1 is rapidly degraded (within 30 minutes) by the ubiquitin proteasome pathway, which subsequently results in an increase in GADD45 α protein expression (54). One possibility is that stress activation of BRCA1 could promote the degradation of ZBRK1 through its E3 ubiquitin ligase activity, relieving *gadd45a* gene repression.

c-Myc

c-Myc is a well-known oncogene that increases its expression in response to mitogens. Its de-regulation prevents cells from exiting the cell cycle and from responding to growth-arrest signals. c-Myc has been shown to regulate many cell cycle control genes including Cdc25a, eIF-4E, C/EBP α , cyclin D1, GADD153, and most recently GADD45 α (56). Marhin *et al*, report that c-Myc activation represses *gadd45a* transcription and that this process requires *de novo* protein synthesis (56). Interestingly, amino acids 106-143 of c-Myc are required for suppression of *gadd45a* transcription, the same region required for cell cycle progression and c-Myc-induced cellular transformation (56). Despite the absence of a canonical c-Myc binding site in the *gadd45a* gene, c-Myc represses its transcription possibly by inhibiting CAAT-binding transcription factors such as the CTF/NF1 or C/EBP family of transcription factors (52, 56). Graunke's LMPCR results in ML-1 cells support Marhin's results, suggesting that C/EBP binds to the *gadd45a* promoter region (48).

NF- κ B

UV treatment has also been shown to activate NF- κ B, a known inhibitor of JNK expression. NF- κ B is thought to cause an inflammatory response and has been shown to be overexpressed in many cancer cell systems. Zerbini *et al* have recently reported that NF- κ B can repress *gadd45a* transcription in an indirect manner by activating c-Myc (57). After inhibiting NF- κ B activation, an increase of approximately 3-fold in GADD45 α and γ mRNA was observed (57). The inhibition of NF- κ B is thought to prevent cell proliferation and increase apoptosis, both of which have been suggested to be functional roles for the GADD45 family of proteins.

FOXO3a

FOXO3a is a member of the forkhead transcription factor family, and its sequence is similar to that of the DAF-16 gene in *C. elegans*, which is required for SOD and catalase transcriptional upregulation. DAF-16 has been shown to be involved in stress resistance and an increased life span, whereas FOXO3a overexpression has been shown to result in G₁ arrest (58). FOXO3a is a downstream target of the PI3K and PKB/Akt pathway, which promotes cell survival. PKB/Akt phosphorylates and inhibits FOXO transcription factors, sequestering the transcription factor in the cytoplasm. Tran *et al*, have shown that inducible expression of an Estrogen Receptor-FOXO3a fusion protein with 4-hydroxytamoxifen can induce *gadd45a* promoter luciferase activity in a Rat-1 cell line (58). Tran and colleagues identified the positions of the forkhead response elements (FHREs) in the *gadd45a* promoter region at -505, -377, and -803, relative to the TSS.

In cells passing through the G₂ phase, FOXO3a was localized to the nucleus. In addition, constitutive expression of FOXO3a led to a G₂/M phase delay as did treatment with a PI3K inhibitor, LY294002. Furthermore, overexpression of FOXO3a managed to repair a UV damaged CMV-luciferase construct in RKO and MEF cells. Tran *et al* also showed that FOXO3a could bind directly to the FHRE2 and FHRE3 elements in the *gadd45a* promoter region (58). All of the FOXO3a effects on G₂ arrest and the UV damaged CMV luciferase construct were diminished in a GADD45 α -/- fibroblast cell line, suggesting that the effects of GADD45 α are at least in part mediated through a transcriptional increase of GADD45 α protein by FOXO3a (58).

Post-Transcriptional Regulation

In addition to transcriptional upregulation, GADD45 α has also been shown to be post-transcriptionally regulated. Rishi *et al* observed a four-fold increase in the amount of GADD45 α mRNA stability in a human breast carcinoma cell line after treatment with the synthetic retinoid CD437 which has been shown to cause G₀/G₁ arrest and apoptosis (59). Rishi's group further established that GADD45 α mRNA stability is dependent upon 45 base pairs of the 5' untranslated region (UTR) of the GADD45 α mRNA (59). Rishi's report is the only published report of post-transcriptional regulation of the GADD45 α protein.

Working Hypothesis

Because GADD45 α is a stress-inducible protein that is responsive to a multitude of DNA damaging agents each with their own mechanism of action, it is reasonable to

suggest that its induction may be related to the production of ROS. UV-irradiation, IR, and sodium arsenite have all been shown to produce ROS in cellular systems and also induce GADD45 α expression (60). We have therefore hypothesized that ROS can act as second messengers to induce the GADD45 α protein and possibly mediate the effects of ROS on the cell cycle, apoptosis, and DNA damage repair. The experimental research presented in this manuscript suggests that arsenite can transcriptionally induce GADD45 α expression via the formation of H₂O₂ through a Fenton-like reaction mechanism.

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SECTION II

Environmental Health Research in the Post-Genome Era: New Fields, New Challenges, and New Opportunities

ABSTRACT

The human genome sequence provides researchers with a genetic framework to eventually understand the relationship between gene-environment interactions. This wealth of information has led to the birth of several related areas of research including proteomics, functional genomics, pharmacogenomics, and toxicogenomics. Developing techniques such as DNA/protein microarrays, siRNA applications, 2-D gel electrophoresis and mass spectrometry in conjunction with advanced analytical software and the availability of internet databases offers a powerful set of tools to investigate an individual's response to specific stimuli. Here, we summarize these emerging scientific fields and techniques focusing specifically on their applications to the complexities of gene-environment interactions and their potential role in environmental biosecurity.

Introduction

Environmental exposure, genetic susceptibility, and age undergo a set of complex interactions to induce human disease (Rall and Groopman, 1994; National Institute for Environmental Health Sciences (NIEHS): Intro to NIEHS, 2002). The prevention and treatment of human disease relies on the ability to understand the exact cause and effect relationship among these factors and to identify which events are involved in the onset and progression of disease. The recently completed draft sequence of the human genome could create seemingly endless opportunities for the delineation of gene-environment interactions. By determining the relationship between environmental exposure and genetic susceptibility, the environmental health research community has the ability to unlock these mysteries.

Since the inception of the Human Genome Project in 1990 (Fink, 1990), new avenues of research such as proteomics, functional genomics, and toxicogenomics have rapidly emerged. In February 2001, two separate drafts of the human genome sequence were published (Venter *et al*, 2001; International Human Genome Sequencing Consortium (IHGSC), 2001). In April of 2003, the IHGSC finally announced the completion of the human genome sequence (Pennisi, 2003; National Human Genome Research Institute, 2003). New techniques including DNA microarrays, small-interfering RNA (siRNA), and protein microarrays have already yielded promising results towards improving health and treating human disease (Heller *et al*, 1997; Novina *et al*, 2002; Belov *et al*, 2001). By integrating information gathered from the human genome sequence, epidemiological surveys, and gene-association/susceptibility studies, it may be possible to determine how genes and environmental stressors are interrelated. These

studies could lead to the generation of novel strategies for treating diseases with underlying genetic components. Here, we discuss advances in the new fields and techniques that have emerged with the promise of a complete human genome sequence and their applications to gene-environment interaction studies.

Environmental Exposure and Genetic Susceptibility

Humans are continually exposed to hazardous agents in the environment through air, water, soil, diet, and the workplace (Churg *et al*, 2003; Tsai *et al*, 1999; Malaka *et al*, 1990; Gambelunghe *et al*, 2003). Previously, there have been many studies focusing on the acute effects of high-dose exposure to environmental agents. However, much less data is available concerning the effects of low-dose chronic exposure to many environmental agents. Chronic exposure to an environmental toxin, even at a low dose, can be just as harmful to an organism as an acute exposure. For example, toxic agents such as cadmium have been shown to accumulate in tissues over time and lead to multiple adverse effects in exposed individuals (Hunder *et al*, 2001; Staessen *et al*, 1999). However, not all individuals who are exposed to an environmental agent will manifest disease, suggesting that many of the effects resulting from a low-dose chronic exposure to an agent may have a complex genetic and/or non-genetic basis. Further complications of exposure assessment include variations in the route of exposure, concentrations of the toxic substance, and the number and length of exposures, rendering each individual's exposure pattern unique.

It has long been thought that the genetic makeup of each individual plays a key role in the cellular response to a toxic agent (International SNP Map Working Group,

2001). There are several types of genetic variations that have been observed in humans, including the loss of one or both alleles for a specific gene, alternative splicing variants, and the presence of single nucleotide polymorphisms (SNPs), which are the most common type of genetic variation. An example of the effect of genetic variation on a cellular process is illustrated by the different expression levels of several cellular antioxidant enzymes in Type 1 Diabetes patients (Hodgkinson *et al*, 2003). When cells and tissues are exposed to a toxic agent such as Cr(VI), the antioxidant enzymes are triggered as a defense system to repair the cellular damage. If, however, a large amount of cellular damage is induced by the Cr(VI) exposure, then the balance between the antioxidant defense mechanisms and the oxidants may be upset. The oxidant-induced damage may be permanent and lead to apoptotic or necrotic cell death (Ye *et al*, 1999). Environmental exposure to agents such as chromium, arsenic, and nickel has been shown to affect several cellular signaling pathways, suggesting that an individual's genetic variation in any gene involved in these pathways could potentially affect susceptibility (Barchowsky and O'Hara, 2003; O'Hara *et al*, 2003; reviewed in Chen and Shi, 2001). As a result, a Type I Diabetes patient that is subsequently exposed to an environmental insult may have a less effective antioxidant defense system than that of a non-diabetic.

There are several ways a toxic agent can elicit a cellular response. For example, an inappropriate activation or modification of proteins involved in the cell survival/DNA repair/apoptotic signaling pathways can lead to increased non-specific tyrosine phosphorylation (Qian *et al*, 2001). Toxic agents, such as nickel, arsenic, and cadmium may also inhibit enzymes responsible for protection from carcinogenic agents (Poirier and Vlasova, 2002). In addition, toxic agents have also been shown to induce mutations,

such as the mutation of two domains of the insulin-like growth factor 1 receptor (IGF-1R) involved in a radio-resistant signaling pathway in mouse embryo fibroblasts (Yu *et al*, 2003). The previous examples suggest that exposure to toxic agents occurs through many different mechanisms, each of which must be fully investigated in order to prevent and/or treat the diseases that result. In addition to the myriad of disease mechanisms that may exist, genetic influences may also play an important role in these mechanisms. Until recently, however, the tools and technology necessary to investigate the genetic influence on diseases of environmental exposure were inadequate. The sequencing of the human genome has provided us with a blueprint to begin studying the gene-environment interactions and the cellular signaling processes that lead to complex, genetically-associated diseases such as Parkinson's, Alzheimer's, and cancer.

Single Nucleotide Polymorphisms (SNPs)

One of the most promising immediate benefits of sequencing the human genome is the current effort to catalog all of the human SNPs. Persons displaying a SNP - a single nucleotide substitution, deletion, or addition in any given gene or regulatory region - are potentially at risk for responding to their environment in a different manner from those who contain the consensus "normal" allele of the given gene. Techniques to determine the effects of these polymorphisms are playing an important role in pharmaceutical research and in the investigation of disease onset. An example is the cytochrome P450 2D6 (CYP2D6) gene encoding debrisoquine 4-hydroxylase, which is involved in the metabolism of many drugs and some neurotoxins. The poor metabolizer

polymorphisms of this gene have been implicated in the pathogenesis of neurotoxin-induced Parkinson's disease (Payami *et al*, 2001).

SNPs are the most common type of genetic variation – purportedly occurring at a rate of 1 per 1300 bases with a grand total of 1.42 million located in the human genome - and are thought to be the most likely candidates for complex polygenic susceptibility to disease (Venter *et al*, 2001; IHGSC, 2001; International SNP Map Working Group, 2001; Wolfsburg *et al*, 2001; Altshuler *et al*, 2000). Several disease genes have already been discovered; the IT15 gene responsible for Huntington's disease, the ADAMTS13 gene involved in thrombotic thrombocytopenic purpura, the lamin A gene (LMNA) which is thought to be the underlying cause of Hutchinson-Gilford progeria syndrome, and the CLN2 gene product associated with late-infantile neuronal ceroid lipofuscinosis (LINCL) (Huntington's Disease Collaborative Research Group, 1993; Levy *et al*, 2001; Eriksson *et al*, 2003; Sleat *et al*, 1997). However, most of these genes are linked to rare conditions in which the mutation of only one gene seems to cause the disease (International SNP Map Working Group, 2001). More complex genetic diseases in which mutations must be present in multiple genes are much more difficult to identify. The identification of such genes will require the characterization of all of the existing SNPs in combination with association studies of individuals at risk (Lander, 1996; Collins *et al*, 1997).

It has already been established that certain SNPs play a pivotal role in the manifestation of disease, either by increasing the risk for disease, by preventing disease progression, or by affecting transcriptional regulation. For example, an ApoE4 SNP has been shown to increase senile plaque density, rendering this SNP a risk factor for Alzheimer's disease (Hofmann, 2001). On the other hand, the deletion of base pair 32 of

the CCR5 co-receptor gene prevents HIV uptake by T4 lymphocytes, rendering any individual possessing this SNP resistant to HIV infection (Lander, 1996; Hofmann, 2001). Finally, cases in which a SNP removes, adds, or alters a transcription factor binding site which lead to a disease phenotype have been documented (Ponomarenko *et al*, 2002; Ponomarenko *et al*, 2003). To date, most of the research of genetic disease has focused on mutations in single genes, mainly because complex genetic interactions are difficult to study (Collins *et al*, 1997). However, by performing gene-association studies utilizing an SNP catalog, researchers can study the complex interactions between multiple genes when exposed to an environmental toxicant (International SNP Map Working Group, 2001; Collins *et al*, 1997).

Many SNP databases are available for public use, including the dbSNP and the SNP Consortium database, both of which currently contain only human SNP data (International SNP Map Working Group, 2001; Smigelski *et al*, 2000; Wheeler *et al*, 2003). Other more specialized types of SNP databases have also been created, including the rSNP_Guide, which allows users to search for novel human transcription factor binding sites created, deleted, or altered by SNPs (Ponomarenko *et al*, 2002; Ponomarenko *et al*, 2003). By utilizing the vast array of information found in such databases, one can begin to explore the probable cause and effect relationship between SNPs and specific gene-environment interactions leading to disease susceptibility.

However, many questions remain to be answered concerning the role of genetic polymorphisms in disease progression, including the following: How do these SNPs facilitate disease? Is it a result of the polymorphic gene's inability to produce its functional protein product? Does the polymorphism destroy some binding sequence

required for a particular signaling pathway? Or does the polymorphism interfere with or negate the immune response to that agent? Can prior exposure create a sensitization effect, i.e. does it influence genetic susceptibility to a second exposure by worsening the effects of subsequent exposure over time? Can genetic susceptibility change or breakdown over time as a person ages? These questions will have to be answered for all of the SNPs involved in a disease in order to fully understand the gene-environment relationship between exposure and disease.

Environmental Genome Project (EGP)

In light of all the questions surrounding genetic susceptibility and environmental exposure, the NIEHS founded the Environmental Genome Project (EGP) in 1997 (EGP – History, 2003). The purpose of the EGP is to increase the available knowledge and information about the effects of environmental exposure and the role genetic variation plays in human susceptibility to disease. Specifically, the EGP focuses on re-sequencing parts of the human genome, looking for polymorphisms that may be involved in genetic susceptibility to a range of diseases (EGP – Overview, 2003). The EGP has identified a group of environmentally responsive genes and categorized them into the following eight groups: cell cycle, DNA repair, cell division, cell signaling, cell structure, gene expression, apoptosis, and metabolism (EGP – Environmentally Responsive Genes, 2003). The goal is to compare and cross-screen the re-sequenced regions of the human genome, analyzing each identified polymorphism and determining whether or not it is functionally significant. Discovering the identity of the functionally significant polymorphisms is crucial to linking genetic susceptibility to specific diseases. Together

with thorough epidemiological surveys, genetic polymorphisms can help determine which environmental and genetic factors may interact to produce disease.

Although studying gene-environment interactions via SNP comparisons is a promising avenue of exploration, some problems may complicate these studies. For instance, disease is not always caused by a single trait; it sometimes involves several traits, and the algorithms available may not be able to deduce a correlation between broad sets of SNPs and environmental factors. Therefore, establishing clear-cut relationships between particular genes and their environmental inducers may not always be possible. These issues will have to be addressed in order to fully understand the mechanisms by which genes and their corresponding SNPs are influenced by their environment.

Bioinformatics and the –omics explosion

As the human genome project neared its completion, the new field of functional genomics began to surface. Functional genomics encompasses the identification and analysis of the entire gene and protein content of an organism (Fields *et al*, 1999; Strausberg and Austin, 1999). Since then, all of the questions originally proposed under the term functional genomics seem to have taken on their own personalities, and have each become their own –omics field. The fields of bioinformatics, proteomics, and structural genomics, are a few of the fields that have evolved in this manner. Developments in these fields will likely facilitate the understanding of the underlying mechanisms of gene-environment interactions and their relationship to human disease.

- *Bioinformatics*

The field of bioinformatics collects, analyzes, and disseminates the raw sequence data of a genome (D Trends, 2003). Researchers are currently trying to create better analytical software and more powerful algorithms to determine which specific sequences correspond to genes or their regulatory regions, and which sections do not play a role in the production of proteins or their regulation. These powerful computer programs will allow bioinformaticians to complete an annotated version of the human genome revealing the number of genes it contains. They will also help to predict the regulatory regions, functionally significant regions, and transcription factor binding sites in unknown genes.

- *Proteomics*

The term “proteome”, coined by Marc Wilkins, refers to the total set of proteins encoded by a particular genome (Wilkins *et al*, 1996). Even before the determination of the complete sequence of the genome, proteomics scientists had already begun to tackle the proteome via techniques involving the coupling of 2D polyacrylamide gel electrophoresis and chemical composition analysis by mass spectrometry (Wilkins *et al*, 1996). By determining the complete contents of the proteome, proteomics scientists hope to discover new protein biomarkers for diseases by examining differences in protein expression levels, post-translational modifications, and the differential expression of proteins between disease and non-disease states. With the development of high-throughput protein analysis methods, such as protein arrays, the identification and cataloging processes can be streamlined. A list of several databases containing

information about protein sequences, 2D gel information, and 3D protein structures is available at the Expert Protein Analysis System (ExPASy) website (Fig. 1) (James, 1997).

- *Structural Genomics*

With the increasing amount of genomic and proteomic data available, structural genomics has found its own niche. The aim of structural genomics is to characterize the structures of representative proteins for each protein family. Several methods, particularly x-ray crystallography and nuclear magnetic resonance (NMR), are used to provide a set of reference structures that will serve to help determine the structures of similar families of proteins. The Protein Structure Initiative (PSI) sponsored by the National Institute of General Medical Sciences has attempted to create a database of the structures of all proteins determined to date (See Fig. 1 - PSI website). Structural genomics scientists can then use the information gained through DNA coding sequences to predict primary protein sequence, which can be further analyzed and compared to known three dimensional structures by computer homology modeling techniques (Yao, 2002).

Computer homology modeling methods play a major role in predicting the tertiary structure of proteins by comparing the sequence of a protein of unknown structure to the sequences of structures that have already been determined via the use of computer databases. Examples of such databases include Entrez's Molecular Modeling Database and the ModBase database (Wheeler *et al*, 2003; Yao 2002; Chen *et al*, 2003). These databases have been employed to determine the physical structure of many proteins and

allow public access to the structure and functional domain data generated for known proteins. The identification of a protein's tertiary structure leading to the recognition of common interaction domains, can offer critical information concerning that protein's function. Recognition of these common binding domains may help to predict novel interactions with other proteins or cellular constituents. Thus, these results could be extrapolated from database resources and used to increase the rate at which candidate proteins can be examined in biological systems - all based solely on their sequence identity and homology to similar proteins.

In addition to comparing the primary sequence of two proteins, the structural databases can be used to compare the tertiary structures of two proteins. Comparing the tertiary structure of proteins may be helpful when comparing two proteins that are functionally equivalent, but evolutionarily diverse (Holm and Sander, 1996). With new technologies developing in the field of structural genomics, the goal of structurally characterizing the entire proteome may soon be attained, allowing researchers to investigate gene-environment interactions at the proteomic level.

Using Bioinformatics to Fuel New Directions in Environmental Health Research

Bioinformatics, proteomics, and structural genomics have produced a large amount of sequence and structural data very quickly. The need for interpretation of this massive quantity of information has led to the development of many new specific disciplines including, but by no means limited to, metabolomics, pharmacogenomics, and toxicogenomics. In addition to these specialized fields, more comprehensive fields such as comparative genomics and systems biology have evolved, both of which attempt to

make more global inferences from a large scale interpretation of all genomes sequenced to date (Yao, 2002).

- *Metabolomics*

The field of metabolomics attempts to decipher the different interactions between signaling pathways by characterizing differences in the metabolome – all of the metabolites present at any given time in a biological sample (Muller and Kersten, 2003). Metabolomic profiles have been used to determine genotypic differences between *Arabidopsis thaliana* plants through the use of neural networks and to study metabolic fluxes in tumor tissue (Taylor *et al*, 2002; Griffiths and Stubbs, 2003). Raamsdonk *et al*, has recently shown that genes exhibiting no overt phenotype can be studied using a method called functional analysis by co-responses in yeast (FANCY) that detects changes in metabolite concentration of a cell rather than an overall growth effect (Raamsdonk *et al*, 2001). Metabolomics studies have also been shown to be useful in the discovery of new toxicity biomarkers, such as the study by Lindon *et al*, in which ¹H-NMR spectroscopy was used to identify novel biomarkers in rat bodily fluids (Lindon *et al*, 2002). EcoCyc, a database that describes the metabolic and signal transduction pathways of *Escherichia coli*, and MetaCyc, which contains metabolic and signal transduction data for many other organisms have recently been developed (Karp *et al*, 2000). Since heart disease has been linked to aberrant cholesterol metabolism, advances in the field of metabolomics could provide major contributions to environmental health researchers investigating the mechanisms responsible for heart disease.

- *Pharmacogenomics*

Pharmacogenomics is a field which aims to identify and characterize the various polymorphisms, including SNPs, present in different individuals that may affect a patient's response to a drug (Altman and Klein, 2002). Examples of an important class of highly polymorphic genes include the cytochrome P450 (CYP450) enzymes, many of which are responsible for the metabolism of an array of currently available pharmaceuticals. Currently, new technical avenues are being explored to determine a patient's genetic compatibility with a drug before a treatment is prescribed. This type of precautionary testing would protect a patient in the event that he or she possessed a polymorphism affecting the metabolism of that drug. Although it has been suggested that less than 1% of SNPs affect the predicted protein coding regions in the human genome (Venter *et al*, 2001), the SNPs that do affect the coding regions can nonetheless have a crucial effect on the treatment of patients. For example, potentially toxic thiopurine drugs, such as 6-mercaptopurine which is metabolized by thiopurine methyltransferase (TPMT), are administered to children with acute lymphoblastic leukemia (Weinshilboum, 2001). Since thiopurines have a very narrow therapeutic index and the levels of TPMT are controlled by a common polymorphism, it is necessary to individualize thiopurine therapy to prevent fatal thiopurine toxicity (Weinshilboum, 2001). Other examples have also begun to pave the way for the individualization of medical treatment. The new Genentech drug Herceptin is being used to treat only the 20-30% of breast cancer patients that overexpress the *her-2/neu* receptor (Service, 2003). Advances in the field of

pharmacogenomics could revolutionize the way a doctor prescribes drugs for his or her patients.

- *Toxicogenomics*

Toxicogenomics is dedicated to the elucidation of the role genes play in response to environmental toxicants and stressors (Hamadeh *et al*, 2002). NIEHS has recently developed the Toxicogenomics Research Consortium (TRC) in conjunction with the National Center for Toxicogenomics Microarray Group (NMG) and has charged them with the specific tasks of: identifying sites of genetic variability throughout the human genome, developing standards for the current microarray technology being used to assess biological responses to environmental stressors/toxicants, and applying this expression technology in a manner that helps to elucidate the mechanisms of toxicity produced in response to environmental exposure (Medlin, 2002; National Center for Toxicogenomics – TRC, 2003). The TRC's overall goal is to study the relationship between environmental exposure and disease, and to show how these gene-environment interactions affect human health. It may be useful to try to develop genome and proteome “fingerprints” of the different toxicants using array technology, in order to thoroughly investigate the mechanism of action of these toxins. The Comparative Toxicogenomics Database (CTD) has been launched in order to provide an annotated guide to toxicologically significant genes (Mattingly *et al*, 2003).

- *Comparative genomics*

Comparative genomics encompasses the effort to compare all known complete genomic sequences to that of the human genome. Complete genomes have now been sequenced for many of the most popular model organisms currently used in research, including the *A. thaliana* plant (*Arabidopsis* Genome Initiative, 2000), the worm *Caenorhabditis elegans* (*C. elegans* Sequencing Consortium, 1998), the fruit fly *Drosophila melanogaster* (Adams *et al*, 2000), the yeasts *Saccharomyces cerevisiae* (Goffeau *et al*, 1996) and *Schizosaccharomyces pombe* (Maier *et al*, 1992), and the laboratory mouse *Mus musculus* (Mouse Genome Sequencing Consortium (MGSC), 2002). The comparative analysis of all of the sequenced genomes allows the determination of which human genes have homologous genes in different model organisms. This allows researchers to make more informed decisions about which model organism to use for specific studies. If a particular drug is known to exert toxic effects in a mouse due to a murine-specific gene, then another model organism could be chosen based on a comparative genomic analysis of several suitable organisms. Thus, some pharmaceutical studies use non-rodent models, such as dogs and primates, in order to assess the toxicological effects of certain pharmaceuticals that are not suitable for the mouse model (reviewed in Smith *et al*, 2001). Comparative genomics can contribute to environmental health research in much the same way, by offering alternative model systems and allowing the elucidation of evolutionarily conserved mechanisms in response to disease. Perhaps similar genetic association patterns may surface between humans and non-rodent organisms that are affected by similar diseases.

- *Comparative Genomics: Sequencing of the Mouse Genome*

In December 2002, the long-awaited draft sequence of the laboratory mouse genome, *Mus musculus*, was finally published (MGSC, 2002). The comparative genomic analyses of the human and mouse genomes may prove to be a major factor in decoding the language of the human genome. Already 342 syntenic segments have been found between both genomes, marked differences in G/C distribution between the two sequences have been discovered, and upon examination of CpG island content, the human genome exhibits about twice as many CpG islands as the mouse genome (MGSC, 2002). Differences in the amount of sequence repeats in the mouse and human genomes is thought to arise from the activity of transposable elements, which appear to be more active in the mouse than in the human (Venter *et al*, 2001; IHGSC, 2001; MGSC, 2002). Since the mouse is the most studied model for human disease, with 99% of its genes corresponding to a human homolog, the comparison of the mouse and human genome sequences will have a significant impact on the future directions of model-driven research (MGSC, 2002). In addition to the identification of homologous gene pairs, the sequence identities of many orthologous disease-associated genes have been compared with an overall amino acid identity approximating 90.3% (MGSC, 2002). These data allow for a more refined extrapolation of mouse experimental data to human disease states.

Currently, there is a Human-Mouse Homology Map sponsored by NCBI that allows the comparison of mouse and human homologous segments of DNA. Several websites also offer access to the entire sequence of the mouse genome, including NCBI's Genome Resources website, the Ensembl Mouse Genome Server, and the USC Genome Bioinformatics website (MGSC, 2002; Wheeler *et al*, 2003). Additional mouse

informatics databases can be found at the Jackson, Harwell, and RIKEN websites (See Fig. 1) (MGSC, 2002).

Aside from comparison applications, the mouse genome sequence will contribute to the generation of new knockout mice. A new transgenic approach by Lois *et al.* shows that an entire mouse can be made to express exogenous genes introduced to the organism via a lentiviral vector system (Lois *et al.*, 2002). This lentiviral vector system could be combined with the new siRNA technology (discussed later) to produce knockout mice in a much more efficient and cost effective manner than traditional methods. It may even be possible to expand the lentiviral technique for use in fully grown mice to knockout embryonic lethal genes in a tissue-specific manner. Techniques of this caliber would provide extremely valuable *in vivo* data on signaling pathways in the mouse that would otherwise be impossible to study.

- *Systems Biology*

In addition to the birth of comparative genomics, the availability of the complete human and mouse genome sequences has also catalyzed the emergence of systems biology, which is the attempt to systematically study all of the physiological processes in a cell or tissue by global measurement of differentially perturbed states. The ultimate goal of systems biology is the integration of data from these observations into models that will eventually represent and simulate all processes of life, including the induction of a diseased state (Ideker *et al.*, 2001; Kitano, 2002). Advances in proteomics could soon make the systems biology approach possible. Examples of systems biology approaches include the galactose metabolism system developed by Ideker *et al.*, the PhysioLab®

technology created by ENTELOS, and the Cardiome Project reviewed by Bassingthwaighe (Ideker *et al*, 2001; Bassingthwaighe, 2000; ENTELOS, 2003).

Current Technology – Global Transcriptional Analysis

The increase in new areas of science prompted by the sequencing of the human genome coincides with a sudden increase in new technology that can help discover links between genes and causation of disease in response to environmental agents. By looking at the expression of a large number of genes at the same time, it is possible to check for global trends of expression specific to disease states. These trends may not be apparent to a researcher using traditional time-consuming methods of gene expression measurement, such as a Northern blot, which relies on *a priori* knowledge of regulation events. Several methods have been developed to assess transcription on a global level, including DNA microarrays, serial analysis of gene expression (SAGE), and massively parallel signature sequencing (MPSS).

- Global Transcriptional Analysis – DNA microarrays

One of the most powerful techniques is the DNA microarray, a high throughput screening method that measures the effects of a stimulus on the mRNA expression of the entire transcriptome in one comprehensive assay (Schena *et al*, 1995; Fodor *et al*, 1993). Microarray technology utilizes a defined set of probes, each of which represents a specific transcript. Each probe is arrayed on glass slides to form a microscopic high-density spot. Total RNA is then isolated from treated and/or control samples and differentially labeled with fluorescent dyes. Next, a mixed population of labeled RNA

targets is hybridized to the set of probes. Finally, the amount of fluorescence is measured by image analysis software and the increase/decrease in gene expression is determined via statistical analysis of fluorescence intensity. Several databases have been created to store microarray data, making it available to the public. These include but are not limited to the Stanford Microarray Database and the NCBI Gene Expression Omnibus database (See Fig. 1).

An attractive advantage to the microarray assay is that the large amounts of expression information obtained through one assay can save a tremendous amount of time by eliminating the need for blind usage of Northern blotting, RT-PCR, western blotting, and enzymatic assays to analyze gene expression. These time-consuming techniques can now be utilized on a smaller scale, as a subsequent validation method, to provide connections for the underlying mechanisms of the signaling pathways (Chin and Kong, 2002). In addition to the standard use of the assay for gene expression, genomic microarrays could potentially be employed to detect single nucleotide polymorphisms (SNPs), since the hybridization between mismatches, even of a 1 base pair magnitude, can be detected upon quantification of the hybridized probe or by enzymatic treatment in certain systems (Kolchinsky and Mirzabekov, 2002; Hacia and Collins, 1999).

As more human genes are identified and characterized, the development of microarrays can be tailored to include specific subsets of related genes that might be of interest to a researcher. One example would be the development of carcinogenesis chips that contain oligo probes specific for all known genes involved in cancer pathology. Toxicology laboratories in the United Kingdom have recently developed a set of toxicology tailored microarrays known as ToxBlot and ToxBlot II, which are specifically

for use in toxicogenomic assays (Pennie, 2000; Pennie, 2002). Subset specific microarray databases, such as the Comparative Toxicogenomics Database (CTD) which contains toxicogenomic microarray data, are also under development (Mattingly *et al*, 2003; Medlin, 2002). These types of specialty-tailored microarrays could become more and more important for the initiation of hypothesis-driven research as we extrapolate information about the individual genes comprising the human genome.

One of the most useful applications of microarrays is the elucidation of uniquely affected transcripts in response to a stimulus, producing a so-called fingerprint pattern. It has recently been shown that microarray data can be useful in diagnosing diseased states in rats by examining the gene expression pattern of white blood cells, producing a fingerprint expression pattern unique to a specific disease state (Tang *et al*, 2001). Previous studies have also shown that different inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease exhibit a differential display of gene expression upon microarray analysis (Heller *et al*, 1997). Moreover, lesions of multiple sclerosis subjected to microarray analysis also produced expression profiles different from that of normal tissue from the same individual (Whitney *et al*, 1999). Most recently, several laboratories have been able to identify a disease state and even predict the prognosis of an individual based on the patient's gene expression profile (Perou *et al*, 2000; Sorlie *et al*, 2001; Brenton *et al*, 2001; Gruvberger *et al*, 2001). These molecular fingerprints of disease produced by microarrays may be able to revolutionize the way we diagnose disease states. Diagnostic microarrays could also be used to identify and assess the severity of environmental exposure even if symptoms have not yet surfaced. With

more research pending, this type of disease diagnosis may become an increasingly powerful tool in the individualization of health care treatment.

In addition to the fingerprint approach to microarray diagnostics, microarrays could also be instrumental for facilitating the identification of biomarkers specific for many different types of disease processes (Gerhold *et al*, 2002; Chin and Kong, 2002). It has been demonstrated that microarrays can detect alterations in gene expression in pathological states, such as microbial colonization of the gut and the onset of inflammation (Heller *et al*, 1997; Hooper and Gordon, 2001). Gel pad microarrays have even been used for identification of all types of pathogens - viruses, bacteria, prokaryotes – and the determination of tuberculosis antibiotic resistance, genetic mutations, SNP markers, and chromosomal rearrangements (Kolchinsky and Mirzabekov, 2002). The identification of new biomarkers will allow for the expansion of the available cache of simple diagnostic tests that can be performed in the laboratories at hospitals, instead of relying on more technically complicated diagnostic methods, rendering faster and more definitive results.

Microarray technology also has practical uses and applications in the environmental health research laboratory. Screening patients for a genetic risk factor is currently feasible only for a small subset of rare diseases that depend on the mutation of a single gene. With the newly emerging genetic technology, it is possible that the list can be expanded to include more diseases, particularly those that are influenced by more than one gene, such as diseases resulting from low-dose, chronic exposure to a hazardous agent. This could potentially save many lives by identifying the diseases much earlier,

allowing doctors to prevent and/or pre-treat diseases before symptoms appear and the disease's course has already damaged the patient's body beyond repair (Grody, 2003).

As there is always a down side to every promising technique, there are also plenty of disadvantages plaguing the microarray. The microarrays currently used in most laboratories are not extremely sensitive and may exhibit probe hybridization technical problems (Kolchinsky and Mirzabekov, 2002). The reliability of the assay is compromised for genes with repetitive sequence elements or nucleic acids that strongly favor a secondary structure (Hacia and Collins, 1999). Additionally, since mRNA is transiently expressed, the expression information obtained through microarray analysis is limited to a "snapshot" of a biological process. Furthermore, early microarray data has not been continuously subjected to a set of globally accepted standards, and therefore much of the microarray data already generated and available in the public databases may be non-comparable or misleading if not examined carefully and analyzed using the appropriate statistical methods (Nadon and Shoemaker, 2002).

Microarray technology has also been criticized for creating a large number of false positives and negatives, for difficulty in reproduction of results between assays and laboratories, and for providing too much information for realistic interpretation. The Microarray Gene Expression Data (MGED) Society has begun to address some of these issues, and is currently trying several approaches such as: developing standards that will help to reduce variability between assays and laboratories which would increase reproducibility, requiring annotation of microarray data provided in databases – the minimal information about a microarray experiment (MIAME) standards, and developing computer software allowing microarray data to be exchanged between laboratories

(Brazma *et al*, 2001). With these improvements looming in the not-so-distant future, microarrays should prove to be an extremely useful tool in the quest for understanding gene-environment interactions.

- *Global Transcriptional Analysis – MPSS and SAGE*

Although DNA microarrays are the most common way to examine transcription on a global scale, MPSS and SAGE offer certain advantages over the DNA microarray. The SAGE technique relies on a set of gene-specific probes complexed with streptavidin beads, each containing a specific tag sequence. The probes and tag sequences are cleaved from the beads by restriction enzymes and combined to form a unique set of markers for each transcript. The transcripts are subjected to PCR and sequenced to determine their identity (Velculescu *et al*, 1995). A SAGEmap database has been developed for the dissemination of SAGE data (See Fig. 1) (Lash *et al*, 2000). Another technique, MPSS, is very similar to SAGE. The designers of MPSS have generated a large set of microbeads, each containing a unique tag attached to a gene-specific probe. The beads containing the tag-probe complex are then mixed with RNA, allowing for hybridization between the RNA and its specific tag-probe complex, and placed into a flow cell where up to a million transcripts can be sequenced simultaneously (Brenner *et al*, 2000).

There are a few advantages to using the SAGE or MPSS approaches over microarrays. One advantage is that SAGE and MPSS are thought to be more sensitive than microarrays. They can detect very low levels of transcripts in a system, whereas microarray experiments may tend to omit some of these low level transcripts. Since the quantitation of MPSS relies on counting the number of beads with a specific tag instead

of hybridizations between a probe and target DNA that may or may not be absolute, the statistical analysis of MPSS is far more robust than that of DNA microarrays (Brenner *et al*, 2000). MPSS is also a much faster technique for global transcriptional analysis than SAGE, since a million transcripts can be sequenced at a time. MPSS also simplifies many of the steps required for preparing a DNA microarray experiment, thus reducing the margin of error in an MPSS experiment.

Current Technology - siRNA

siRNA can also be very useful for the elucidation of signaling pathways induced by different environmental agents, particularly now that the complete human genome sequence is available. RNA interference (RNAi) is a naturally occurring mechanism in which the presence of dsRNA can lead to gene silencing through siRNA intermediates. siRNAs are 21-23 base pair sequences that are created from larger dsRNA molecules by an enzyme of the RNase III family called Dicer. The siRNA fragments then associate with a nuclease complex termed the RNA-induced silencing complex (RISC). RISC unwinds the siRNA fragments and allows the single-stranded fragment to target complementary RNA inside a cell, which somehow activates the nuclease activity of the RISC complex (reviewed in Ambion, 2003).

RNAi was first discovered in the worm, and has been a commonly utilized technique in the invertebrate world (Fire *et al*, 1998; Zamore, 2001). Until recently, RNAi was thought to be an application that could only be used in invertebrate studies, since a double stranded RNA greater than 30 base pairs in length seemed to activate the PKR kinase anti-viral mechanism and induce apoptosis in mammalian cells (Ambion,

2003; Zamore, 2001). However, it was recently discovered that the 21-23 nucleotide siRNA fragments could bypass the apoptotic mechanism and produce a gene-silencing effect in mammalian cells (Elbashir *et al*, 2001). Although the precise details of the mechanism of siRNA-silencing are not entirely known, it is still an extremely useful laboratory technique. The short siRNA fragments can be synthetically produced by silencing vectors, and transfected into mammalian cells, resulting in a decrease of a specific protein from the system (Elbashir *et al*, 2001). An advantage of siRNA over the conventional practice of over-expressing dominant-negative mutants in cellular systems, is that the siRNA removes endogenously expressed mRNA. This method is also much easier than creating knockout mutations in cell lines, since it requires only the synthesis of siRNA fragments and a transfection.

Several labs have recently suggested that siRNA technology could have clinical applications, such as the inhibition of HIV-1 replication, in addition to its research applications (Jacque *et al*, 2002; Novina *et al*, 2002). Capodici *et al*. have shown that they can inhibit HIV-1 infection and replication by transfecting primary CD4⁺ T cells with HIV-1 specific siRNA (Capodici *et al*, 2002). In addition, Qin *et al*. have developed a lentiviral-mediated delivery system for siRNA complementary to CCR5 mRNA, the main HIV-1 co-receptor in primary CD4⁺ T cells (Qin *et al*, 2003). This application seems to lead to the prevention of HIV-1 infection, mimicking the natural immunity to HIV-1 seen in individuals who lack both CCR5 co-receptor genes (Qin *et al*, 2003; Dean *et al*, 1996). Future siRNA research utilizing approaches such as these, could lead to the use of siRNA and retroviral gene therapy applicable not only to HIV-1 infection, but to environmentally-induced disease states as well.

siRNA could become a very powerful tool for the environmental health research community. Once the sequence of a particular gene that responds to an environmental toxin is known, siRNA can be easily exploited to reveal the consequences of removing that protein from a cellular system. Other suggested uses for the siRNA technology that could benefit environmental health research include the development of siRNA chips. These chips would consist of an array of unique siRNA probes fixed to a tissue culture slide. Cells could then be grown on these chips to show a range of siRNA suppression based on the siRNA vector the cell takes up, allowing for a high-throughput format for siRNA studies (Zamore, 2001). In addition, a new type of ssRNA, termed microRNA (miRNA) or small temporal RNA (stRNA), is thought to function in translation suppression. These ssRNA molecules may be useful for genetic-silencing experiments as well (Zamore, 2001; Dennis, 2002).

Emerging Technology – Peptide/Protein Microarrays

With the recent push towards high throughput technology, it only seems natural that peptide arrays would follow the advent of DNA microarrays. The set up of a peptide microarray is very similar to that of a DNA microarray. In both cases, a set of unique probes, or capture agents, are attached to a glass slide, or some other form of solid support system. The main difference between the two assays is the type of capture agent that is attached to the slide. In the protein microarray, the capture agent can range from antibodies, epitopes, and small molecules to sets of recombinant proteins (Tyers and Mann, 2003). On the contrary, cDNA or oligonucleotide probes are used in DNA microarrays. The different probes in a protein microarray are chosen depending on what

type of assay the researcher wants to run. Examples of peptide microarray assays include binding assays, enzymatic activity assays, protein identification assays, and cell adhesion assays (Reimer *et al*, 2002; MacBeath, 2002).

One potential application of a protein microarray is the enzymatic kinase assay. In this assay, a purified ^{32}P -labeled kinase can be applied to an array utilizing peptides as capture agents. Each protein probe that is phosphorylated by that specific kinase would then bear the radio-label (MacBeath and Schreiber, 2000). Alternative uses of the protein microarray include the use of antibody probes, which could detect specific proteins from a mixed solution of total protein isolated from a test subject (Belov *et al*, 2001). The development of yet another peptide microarray assay includes the placement of different proteins onto the glass slide and washing the slide with a mixture of differentially fluorescent labeled proteins in order to determine protein binding interactions (MacBeath and Schreiber, 2000). Assays such as these could advance the discovery of biomarkers important for the identification of diseased-state phenotypes. These assays may also help elucidate exciting new protein-protein interactions.

Although peptide microarray technology is still in its infancy, the advantages are apparent. In particular, the arrays offer a high-throughput advantage over traditional methods such as yeast-two hybrid screens, fluorescence resonance energy transfer (FRET), and the traditional biochemical assays coupled to mass spectrometric analysis (reviewed in Phizicky *et al*, 2003). Protein microarrays can also help fill in the gaps left by DNA microarrays, since DNA arrays are indifferent to some forms of genetic regulation, such as post-translational regulation and protein degradation. The development of solid protein profiling techniques and applications would allow

researchers to supplement the information gained through DNA microarray studies. In addition, a global analysis of the entire proteome could lead to the quick discovery of protein-protein interactions that may have taken years to notice with a small scale approach.

Currently, however, it is not yet technically possible to determine the entire content of a proteome with a protein microarray. Several complicating factors, such as the existence of unknown differential splice variants and post-translational regulation events undergone by many proteins, make it difficult to develop capture agents for these currently unknown proteins. Difficulties also arise during the production of the capture agents that are needed to specifically interact with only a single protein. Another disadvantage of protein arrays that measure specific binding to a set of capture agents is that attachment to the slide must not alter the structure or function of the capture agents (MacBeath, 2002). Yet another issue relates to the difficulty of synthesizing the proteins on the glass slide, possibly creating a bias as to which proteins get studied, e.g. the easiest ones to synthesize on the slide. Other concerns have been voiced about the difficulties of miniaturizing the protein microarrays while maintaining sensitivity and the need for algorithms that are advanced enough to handle a protein array format.

One of the most important issues of implementing protein microarrays is the difficulty in producing large numbers of highly pure recombinant proteins. However, recent advances in protein complex purification involving the tandem affinity purification method combined with RNAi (known as iTAP) by Forler *et al.* are a step in the right direction (Forler *et al.*, 2003). The sequence of the human genome may also help to remediate this shortcoming by allowing the development of a perfectly normalized

human cDNA clone library spanning the entire proteome. In fact, the FlexGene Consortium has recently announced their mission to develop a complete cDNA collection specifically for this purpose (Tyers and Mann, 2003).

New advances in nanotechnology may also help to overcome many of the technical issues with the newly developing protein arrays. Some of these new advances include the research of Ouyang and colleagues, who have demonstrated a soft-landing mass-spec technique to separate protein mixtures and spot proteins in a microarray format. An array of proteins was created by their soft-landing technique that allowed proteins to retain their biological activity (Ouyang *et al*, 2003). Other advances in nanotechnology that may assist protein array implementation include the development of new protein capture agents, such as the mixed-element capture agents (MECAs) developed by Bachhawat-Sikder and Kodadek. MECAs comprised of two small proteins attached together with a variable linker region and were shown to bind their target molecules with high affinity and sensitivity, making them good candidates for protein array capture agents (Bachhawat-Sikder and Kodadek, 2003).

Despite the immaturity of peptide microarray technology, results from primitive protein array assays have already indicated the usefulness and incredible versatility of the assay. Analogous applications to those of DNA microarrays, such as using protein profiling to obtain a characteristic fingerprint of a single disease, have already been developed. In fact, protein microarray profiling has recently been used to fingerprint different leukemia types, which may someday become a widely used diagnostic tool (Service, 2003; Belov *et al*, 2001). In addition to fueling new research applications, protein profiling could soon take its place next to the DNA microarray as a new

diagnostic tool. Together with DNA microarray technology, peptide microarrays may also help to revolutionize many aspects of the health care field, including applications to diagnostics and assistance with the personalization of disease treatment.

Biosecurity and Bioterrorism

We have all probably noticed the increased attention focused on bioterrorism in the United States since the attacks of September 11, 2001 and the subsequent reports of inhalation anthrax in October, 2001 (Jernigan *et al*, 2001). Traditionally, biowarfare research has mainly been conducted by military scientists in government facilities. However, in 2003 alone, approximately \$1.75 billion has been poured into biological weapons research, much of which is being allocated to the private and academic sectors to develop biowarfare research programs and facilities (Fauci, 2002). Biosecurity and bioterrorism are a definite concern for the environmental health research community. In response to the increasing threat of the use of biological and chemical weapons in a terrorist attack, the Centers for Disease Control and Prevention (CDC) has compiled a list of biological agents that would most likely be used in a terrorist attack (Rotz *et al*, 2002) (See Table 1). The dissemination of aerosolized particles of many of these agents into the environment is considered to be extremely dangerous. Therefore, it is the responsibility of environmental health researchers to help develop new methods of detecting chemical-biological (CB) agents and aide in the advancement of toxicology and genetic interaction studies pertaining to novel vaccines and treatments.

Preparedness in the Case of a CB Attack

In the past, emphasis has been placed on the ability of health care facilities to communicate with each other and coordinate treatments in the event of a CB attack. Such emphasis has prompted the development of many new training and preparedness programs, including preparedness programs at the state and federal levels and non-governmental professional programs, such as the Clinicians' Biodefense Network (CBN). The CBN aims to link clinicians to health experts and biomedical researchers, allowing for rapid communication and the distribution of updated information concerning biodefense during times of crisis (Radonovich, 2002). However, education and preparedness of healthcare workers is not enough to protect the mass population.

Today, more emphasis has been placed on developing countermeasures in the event of a biological attack, and there has been an overwhelming push by the government and the public for further research into biosecurity efforts. It thus follows, that the determination of the gene-environment interactions that can occur in response to biological or chemical weapon attacks should be a major priority in the environmental health research community. The development of rapid and standard laboratory tests detecting exposure to a specific kind of CB agent that could be widely and economically distributed to health care facilities would greatly enhance our ability for the early detection and response to certain types of potential attacks. Candidate assays for these tests include the identification of specific biomarkers for exposure to CB weapons. Biomarkers could easily be assayed for changes in exposed victims and would greatly aid in the early clinical recognition of such an attack. With the recent anthrax attacks, an early screening method for potential biomarkers specific to anthrax infection applied to

all who were suspected of anthrax exposure would have improved the ability of the nation's public health system to respond, and in turn could have possibly saved lives and prevented the needless distribution of large amounts of Cipro (ciprofloxacin, the antibiotic used to treat anthrax infections) (Jernigan *et al*, 2001; Rosenberg, 2003). With the information gained from the completion of the human genome sequence, and the multitude of emerging technologies available, there are many opportunities for the exploration of bioterrorism countermeasures.

Preparedness in Case of a CB Terrorist Attack-Mass Vaccination

Many of the vaccines and treatments used to prevent the spread and disease manifestations of potential bioterrorism agents were manufactured over 20 years ago; in the case of the smallpox virus, the vaccine used to eradicate the disease in 1980 is the only currently available vaccination (Lofquist *et al*, 2003; Fauci, 2002; WHO, 1980). Previous data has suggested that a smallpox outbreak would spread slowly (Eichner and Dietz, 2003). The current recommended public health response to a smallpox outbreak is a ring vaccination strategy in which individual cases are isolated, quarantined, and their contacts vaccinated (Whitley, 2003). However, some scientists have shown that this approach may not be as effective as an immediate mass vaccination in the event of a biological attack on an urban center (Kaplan *et al*, 2002), suggesting that new strategies should be considered.

Several setbacks have surfaced during discussions concerning vaccination to protect the mass population in the event of a biological attack. One of the most important is the lack of interest from pharmaceutical companies to develop and produce vaccines

for biological warfare agents. Most of the larger companies are unwilling to develop these vaccines because of financial concerns, liability issues, efficacy questions, and regulatory limitations (Rosenthal and Clifford, 2002; Langford and Myers, 2002).

Another concern is the high rate of complications in the vaccines that have been licensed and are used exclusively in the military, but not in the general public. The high occurrence of adverse effects in such a small population (reported to be >30% in some instances) suggests that the benefits of vaccination in the case of a potential threat may not outweigh the risks of vaccine complications (Frey *et al*, 2002). However, identification of SNPs in certain populations via microarray analysis may help to determine which subset of the population should not be vaccinated in preparation for a biological attack. The identification of these individuals would result in a decrease in the number of unnecessary complications/deaths that could occur in the event of a mass vaccination, should a bioterrorism situation arise. Other measures would need to be taken to protect these individuals. Although the United States currently has licensed vaccines for smallpox and anthrax, it does not have these for many of the other agents listed as potential biological warfare agents by the CDC and researchers are working fervently to develop them (See Table 1). Newly developed Ebola vaccines have recently been shown to protect mice, guinea pigs, and non-human primates from lethal doses of the virus (Vanderzanden *et al*, 1998; Xu *et al*, 1998; Sullivan *et al*, 2000).

In addition to vaccination, other methods of preparedness have been discussed. Recently, a set of guidelines has been published by the CDC's National Institute for Occupational Safety and Health (NIOSH), outlining the procedures building officials should follow to protect private, public, and government buildings from chemical,

radiological, and biological terrorism (NIOSH, 2002). A second set of guidelines has been published by the Office of Health and Safety to ensure laboratory security and emergency response preparedness for laboratories that work with CB agents deemed fit for terrorist attacks (Richmond and Nesby-O'Dell, 2002). In order to further protect building environments from a CB attack, engineers have also been trying to develop a set of biomaterials that can metabolize CB agents. The approach is designed to allow the incorporation of these materials into equipment, such as the countertops of an office building or the sorting machines used in post offices (reviewed in Russell *et al*, 2003). The development of such materials combined with the protective measures outlined by NIOSH and OHS could allow for widespread protection from attacks in almost any building or environment, while diminishing public fear of an attack.

Early Detection of CB Agents in the Environment

Although much has been discussed concerning early disease recognition, improved surveillance/communication methods, and the limitation of potentially sensitive scientific information (Zilinskas and Tucker, 2002; Atlas *et al*, 2003; Chyba, 2001; Kwik *et al*, 2003), less has been suggested concerning our ability to detect a CB attack before clinical disease is recognized in victims. The early detection of the agent itself in the environment, which could help prevent the spread of the infection in the case of a biological attack and limit the number of victims affected by the agent, would be more advantageous than the early detection of diseases caused by these agents.

One of the most effective ways to deliver a biological weapon is the release of aerosolized particles into the environment. Implementing a biological agent monitoring

system that can detect a CB agent by sampling the air would greatly enhance our ability to evacuate enclosed areas deemed ideal for CB attack (Wyatt, 2002). Testing has recently begun at San Francisco International Airport for one of the first CB agent defense systems (Larkin, 2003). The system, known as PROACT (Protective and Response Options for Airport Counter-Terrorism) and produced by Sandia National Laboratories in Livermore, CA, is designed to monitor the air at large indoor public meeting areas for CB agents as a measure to deter chemical attacks such as the 1995 sarin attack in the Tokyo subway system (Frischknecht, 2003; Larkin, 2003). Details of the mechanism of action of the device have not yet been disclosed. However, if it proves to be reliable, the device may spread to other airports across the nation.

Immunological methods to detect biological warfare agents are also being investigated for use in an early warning detection system. A miniature biochip system based on an ELISA assay has recently been developed by Stratis-Cullum *et al.* In this system, air samples containing aerosolized spores of *Bacillus globigii* were applied to a miniature ELISA biochip, where a novel alkaline phosphatase substrate, dimethylacridinone phosphate (DDAO-P) was cleaved by an alkaline phosphatase (AP) enzyme (Stratis-Cullum *et al.*, 2003). The DDAO formed as a result of AP cleavage can be excited by a miniature diode laser and the voltage output read on a laptop computer. Although this highly sensitive, portable device was used for the detection of *B. globigii* spores, it could be used to detect other aerosolized forms of biological weapons.

Another immunoassay, the multiplexed liquid array, has been shown to be useful in the simultaneous detection of many different biological agents (McBride *et al.*, 2003). McBride and colleagues used a set of polystyrene microbeads conjugated to biowarfare

agent-specific antibodies, each of which displays a unique spectral pattern. Once the microbeads are exposed to antigen, a secondary antibody conjugated to the fluorescent reporter phycoerythrin (PE) is applied, and the beads are assayed in a flow cytometer. The presence of the unique spectral pattern detects the type of antibody that reacted with a biowarfare agent, while the PE is detected simultaneously to quantify the amount of agent present in the air. These types of assays are candidates for use in early warning detection systems because they are rapid, easily detected, and are part of a portable unit.

Environmental Health Research, the Human Genome Project, and Biosecurity

The DNA microarray and protein microarray applications discussed previously can also be applied to bioterrorism. These techniques can be useful in leading us towards unique physiological responses that may occur when an individual has been exposed to these agents, providing scientists with a much narrower spectrum of therapeutic targets to focus on during their research. The genomes of several potential biowarfare agents have already been sequenced, including that of *Bacillus anthracis* (Read *et al*, 2003).

Microarrays have already been used to study the effects of the *B. anthracis* virulence factors *atxA* and *acpA*. These studies suggest that *atxA* is a global regulator of many new genes that could potentially exhibit virulence effects (Bourgogne *et al*, 2003). By treating human protein arrays with a mixture of proteins from a biowarfare agent, one could infer interactions between the proteins of the agent and human proteins, leading to new drug targets, new signaling pathways, and new routes of infection. Implementing the use of databases containing collections of array data would allow the expression effects of these agents to be cross-referenced, possibly leading to the identification of a few candidate

therapeutic targets that could be used for the treatment of diseases induced by CB agents. As suggested earlier, microarrays may also prove to be important in the identification of novel or weaponized infectious agents. Microarrays played a large role in the identification and characterization of the Severe Acute Respiratory Syndrome (SARS) coronavirus that plagued China, Canada, and Vietnam, among others (Ksiazek *et al*, 2003). Additional applications include the use of microarrays to differentiate between a large spectrum of possible viral infections and to determine the genotype of these pathogens (Wang *et al*, 2002).

Novel Treatments in a CB attack

In addition to being concerned about preparedness in the case of an attack, novel treatments of diseases caused by such CB agents should be thoroughly investigated. One of the easiest ways to weaponize a bacterial strain for use in a biological warfare attack is to engineer antibiotic resistance genes into the strain. Since this is not an extremely difficult task, the need for non-antibiotic treatment of weaponized strains of biological agents is an important matter. A novel approach to treating victims of a CB attack, developed by Fischetti, is the use of purified bacteriophage lysins to specifically lyse targeted bacteria, including those exhibiting antibiotic resistance. Fischetti's group demonstrated that PlyG, a gamma phage lysin specifically targeting *B. anthracis*, effectively killed spores *in vivo*. PlyG treated mice had a 72% survival rate versus a 10% survival rate for untreated mice (Fischetti, 2003). In addition to the bacterial lysin approach, it may also be possible to deliver siRNA through a viral gene therapy

mechanism as a therapeutic countermeasure to interfere with an agent's ability to replicate.

Future Directions

In order for scientists to get the maximum benefit from the current technology available, significant technological and organizational improvements must be made. Advanced software is required for bioinformaticians to accurately recognize common binding domains, promoter regions, and transcriptional regulatory elements. The development of user-friendly, organized databases providing DNA and RNA sequences, protein interactions, protein structures with correct annotations, microarray data, and a variety of animal genome sequences appear necessary to solve the puzzle of signaling pathways, which can have profound effects on human disease states.

There are also many different forms of data that need to be explored on a multi-level scale. Genomes, transcriptomes, proteomes, and metabolomes are not separate entities and each -omic level must be taken into consideration when integrating the different types of information available. For example, many proteins are multi-functional and may act at the level of transcription as well as participating in a cellular signaling cascade. We must be able to view each piece of information from a global standpoint.

With the surge in the development of new techniques and fields of expertise, the environmental health research community can use the information provided by the genetic code to its advantage. The improved identification of environmentally hazardous agents via gene and protein expression analysis as a diagnostic tool in health care facilities could soon become a reality, allowing for better assessment of environmentally

induced health risks and ultimately more accurate treatments. The elucidation of biological responses to toxic environmental agents and the mechanisms by which these agents cause cellular damage may aid in developing new drug targets and/or novel therapeutic options. Transcriptome expression patterns could soon be identified for individual toxicants and stressors, which may improve exposure assessment methods. Additionally, the discovery of new biomarkers activated in response to environmentally hazardous agents and the use of array expression analysis may soon be indispensable tools for diagnosing a disease, determining its stage, predicting its course, and thus dictating its treatment on an individual basis. The identification of susceptibility factors through SNP screening could lead to a better assessment of risk factors for potentially susceptible individuals and possibly even lead to prevention of adverse effects or even some diseases altogether.

Concluding Remarks

In conclusion, the elucidation of a first draft of the human genome sequence has provided the environmental health research community with a wealth of information and a genetic framework that can greatly contribute to our understanding of gene-environment interactions. Determining the actual extent of polymorphisms and assigning function to the remaining 40% of predicted proteins with unknown molecular function (Venter *et al*, 2001), are only a few examples of the challenges still ahead. However, with the current information available and by using these new techniques and the expertise of those involved in the emerging fields of environmental health research, we can close in on our common goal of providing the world with better treatments and

knowledge to prevent the atrocious diseases that can result from exposure to harmful environmental factors.

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Figures and Tables

Figure 1: Webguide to Bioinformatics Sites and Useful Database Links

Bioinformatics Sites

HGP Timeline	http://www.ornl.gov/TechResources/Human_Genome/project/timeline.html
Environmental Genome Project	http://www.niehs.nih.gov/envgenom/home.htm
NCBI Gene Expression Omnibus Database	http://www.ncbi.nlm.nih.gov/geo
FlexGene Consortium cDNA database	http://www.hip.harvard.edu
NCBI Genome Resources	http://www.ncbi.nlm.nih.gov/genome/guide/mouse/
University of Southern California Genome Bioinformatics	http://genome.ucsc.edu
Cancer Genome Anatomy Project	http://cgap.nci.nih.gov
HyperCLDB - Cell Line Database	http://www.biotech.ist.unige.it/cldb/indexes.html
Gene Ontology Consortium	http://www.geneontology.org

SNP Sites

dbSNP	http://www.ncbi.nlm.nih.gov/SNP
SNP Consortium Database	http://snp.cshl.org
rSNP_Guide	http://www.mgs.bionet.nsc.ru/mgs/systems/rsnp/
Debbie Nickerson Lab SNP catalogs	http://droog.mbt.washington.edu

Proteomics and Structural Genomics Sites

Entrez Molecular Modeling Database	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Structure
ModBase Database	http://guitar.rockefeller.edu/modbase
Ann Stock Lab - Structural Genomics Links	http://stock.cabm.rutgers.edu/links/
Protein Structure Initiative	http://www.structuralgenomics.org/
ExpASy Molecular Biology Server	http://us.expasy.org/
RCSB Protein Data Bank	http://www.rcsb.org/pdb/
Danish Cancer Society - 2D Protein Database	http://proteomics.cancer.dk/
Philip Andrews Lab - Proteomics Links	http://www.proteome.med.umich.edu/andrewslab/links/proteomics.html
TRANSFAC - Transcription Factor Database	http://www.gene-regulation.com/

Pharmacogenomics Sites

HGP – Pharmacogenomics	http://www.ornl.gov/TechResources/Human_Genome/medicine/pharma.html
Pharmacogenomics and Pharmacogenetics Knowledge Base	http://www.pharmgkb.org/index.jsp

Toxicogenomics Sites

Toxicogenomics Research Consortium
National Center for Toxicogenomics Microarray Group
Toxicology and Environmental Health

<http://www.niehs.nih.gov/dert/trc/home.htm>
<http://www.niehs.nih.gov/nct/nmc.htm>
<http://www.sis.nlm.nih.gov/Tox/ToxMain.html>

Comparative Genomics and Systems Biology Sites

Harvard-Lipper Center for Computational Genetics
Princeton Model Organism Virtual Libraries
ENTELOS - Virtual Obesity Model
Virtual Heart Project

<http://arep.med.harvard.edu>
<http://www.molbio.princeton.edu/scientific.php>
www.entelos.com
www.physiome.org

Mouse Genome Informatics

Ensembl Mouse Genome Server
The Jackson Lab - Mouse Informatics
Harwell Mouse Informatics
RIKEN Mouse Informatics

http://www.ensembl.org/Mus_musculus/
<http://www.informatics.jax.org>
<http://www.har.mrc.ac.uk/>
<http://genome.rtc.riken.go.jp/>

Microarray Sites

The Brown Lab Microarray Database
Microarray Gene Expression Database (MGED)
Stanford Microarray Database
Microarray Gene Expression Data (MGED) Society
Telechem International E-Library
The Institute for Genomic Research (TIGR)
TIGR Microarray Software Suite
TIGR Gene Indices
TIGR Microarray Tools
MIAMExpress
The Churchill Lab Microarray Statistical Software
MIAME Checklist

<http://cmgm.stanford.edu/pbrown/index.html>
<http://www.mged.org>
<http://genome-www5.stanford.edu/MicroArray/SMD>
<http://www.mged.org>
<http://arrayit.com/e-library/>
<http://www.tigr.org>
<http://www.tigr.org/software/tm4>
<http://www.tigr.org/tdb/tgi>
<http://pga.tigr.org/tool.shtml>
<http://www.ebi.ac.uk/miamexpress/>
<http://www.jax.org/staff/churchill/labsite/software/index.html>
http://www.mged.org/Workgroups/MIAME/miame_checklist.html

SAGE and MPSS Sites

NCBI's SAGEmap
Arabidopsis MPSS Database

<http://www.ncbi.nlm.nih.gov/SAGE/>
<http://mpss.udel.edu/at/java.html>

Table 1: CDC’s List of Potential Biological Warfare Agents¹

Category A	Category B
<i>Bacillus anthracis</i>	<i>Brucella</i> species
<i>Clostridium botulinum</i> toxin	<i>Clostridium perfringens</i> ε-toxin
<i>Yersinia pestis</i>	Food Safety Threats (i.e. <i>Salmonella</i> species, <i>E. coli</i> O157:H7, and <i>Shigella</i>)
<i>Variola major</i> (Smallpox)	<i>Burkholderia mallei</i>
<i>Francisella tularensis</i>	<i>Burkholderia pseudomallei</i>
Viral Hemorrhagic fevers (i.e. Ebola, Marburg, Lassa, and Machupo)	<i>Chlamydia psittaci</i>
	<i>Coxiella burnetii</i>
	Ricin toxin
	Staphylococcal enterotoxin B
	<i>Rickettsia prowazekii</i>
	Viral encephalitis (i.e. alphaviruses)
	Water Safety Threats (i.e. <i>Vibrio cholerae</i> , and <i>Cryptosporidium parvum</i>)

* Category C agents include any emerging infectious disease including Nipah virus and hantavirus.

¹Adapted from the CDC website at www.bt.cdc.gov/agent/agentlist-category.asp

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SECTION III

Molecular mechanisms of metal toxicity and carcinogenesis

Abstract

Chronic exposure to many heavy metals and metal-derivatives is associated with an increased risk of cancer, although the mechanisms of tumorigenesis are largely unknown. Approximately 125 scientists attended the 3rd Conference on Molecular Mechanisms of Metal Toxicity and Carcinogenesis and presented the latest research concerning these mechanisms. Major areas of focus included exposure assessment and biomarker identification, roles of ROS and antioxidants in carcinogenesis, mechanisms of metal-induced DNA damage, metal signaling, and the development of animal models for use in metal toxicology studies. Here we highlight some of the research presented, and summarize the conference proceedings.

Introduction

The 3rd Conference on Molecular Mechanisms of Metal Toxicity and Carcinogenesis was held at the National Institute for Occupational Safety and Health (NIOSH) in Morgantown, West Virginia. Over 125 participants from 13 different countries gathered at NIOSH to present their research on September 12–15, 2004. This conference was the third meeting of metal researchers in the past 5 years, with previous conferences convening in 2000 and 2002. Major areas of research emphasized at the conference included metal epidemiology, identification of biomarkers, reactive oxygen species (ROS) production, antioxidant effects, induction of DNA damage/lesions, metal-induced signaling pathways, and animal exposure studies. Here, we summarize the conference proceedings and examine the future directions of research concerning the molecular mechanisms that facilitate metal-induced carcinogenesis.

Epidemiological implications of metal exposure and the search for reliable biomarkers

Many metals are considered essential trace elements and must be present in low concentrations in the human body in order for normal cellular function. However, altered concentrations or transition states of metals in the body are thought to lead to a wide range of deleterious conditions, especially an increase in cancer incidence. Increased metal exposure in humans can occur via ingestion, inhalation, dermal contact, and occupational exposure [1–4]. Although correlations between metal exposure and cancer are well documented, more research is still needed in order to determine the exact mechanisms of metal-induced carcinogenesis. Much of the metal toxicology and carcinogenicity data available are acquired in animal model systems, with few studies

representing the effects of metals in humans. Therefore, several groups at the conference focused their research on the link between the level of metals in the human body and carcinogenesis. Puneet and colleagues presented work examining the role of metals in gallbladder carcinoma. Using atomic absorption spectrometry, they showed that the bile of gallbladder carcinoma patients contained higher concentrations of cadmium (Cd), chromium (Cr), and lead (Pb) than patients with other non-cancerous gallbladder diseases, such as gallstones.

Additionally, Puneet's group observed an increase in the expression of the metal-exposure protection protein metallothionein (MT) in 70% of the gallbladder carcinoma patients. These results suggest that the body may concentrate these metals in the hepatobiliary system and lead to gallbladder carcinoma. This group also examined the lipid peroxidation profiles of gallbladder carcinoma patients in order to determine if a free radical-induced mechanism might be involved. They found a significant increase in the amount of a major lipid peroxidation product, 4-hydroxynonenal (4-HNE), in gallbladder carcinoma patients compared to patients with other non-cancerous gallbladder diseases. These results suggest that the formation of lipid peroxidation products in the gallbladder, induced by metal exposure and free radical generation, may be an underlying cause of tumorigenesis.

Many metals must be controlled by a tightly regulated homeostasis. Two such examples are copper (Cu) and iron (Fe), the accumulation of which results in oxidative stress-induced cellular damage [5, 6]. Wilson's disease (WD) and primary hemochromatosis (PH) are metal storage diseases which are associated with DNA damage in the liver. In WD, a defect in a Cu-transporting ATPase results in the

intracellular accumulation of Cu [7]. In PH, Fe accumulates in the liver because of a mutation in the *hfe* gene, which is responsible for mediating the effects of the transferrin receptor [8]. Patients with both WD and PH are shown to exhibit increased oxidative stress-induced etheno-DNA adducts [9]. Vinyl chloride, urethane, and 4-HNE produce etheno-DNA adducts as well [10–12]. These adducts can subsequently lead to the formation of strong miscoding lesions and cause mutations and/or genomic instability in patients suffering from WD and PH. Nair and Bartsch further examined the correlation between the formation of 4-HNE-induced DNA adducts and susceptibility to the development of liver cancer. They found that both WD and PH patients exhibiting high 4-HNE-induced DNA adducts were the most susceptible to the development of liver cancer, particularly those patients suffering from PH.

Conversely, the dietary deficiency of many micronutrients, such as certain trace metals, has also been associated with an increased risk of cancer [13–16]. Ansari *et al.* presented data suggesting that significantly lower levels of selenium (Se) and zinc (Zn) were found in the serum, biliary fluid, and gallbladder tissue of gallbladder carcinoma patients when compared to healthy individuals. Serum manganese (Mn) was also reduced in gallbladder carcinoma patients, leading Ansari's group to conclude that a deficiency in these trace elements is also associated with gallbladder carcinoma.

Since animal studies assessing the effects of metal exposure can often be difficult to interpret and inconclusive due to the role of some metals as co-carcinogens, reliable biomarkers are needed in order to assess metal exposure in humans. A good candidate for a human biomarker must be specific to the effects of the causal agent and easily quantitated and/or assayed for its presence in a noninvasive manner. Several studies

presented at the conference examined the reliability of biomarkers for metal exposure. Using atomic absorption spectrometry, Rahnama *et al.* analysed the Zn, Cu, Mn, and Fe content of saliva and serum from patients with squamous cell carcinoma (SCC) of the oral cavity. SCC patients exhibited increased concentrations of Cu, Mn, and Fe in their blood and increased concentrations of all four metals examined in saliva when compared to healthy individuals. These results suggest that analysis of saliva may be an easy and effective way to detect SCC of the oral cavity.

In order to identify specific biomarkers of mercury (Hg) poisoning Song *et al.* examined cases of peripheral neuropathy in Hg-exposed patients. Levels of selected urinary proteins were measured in patients with acute or chronic Hg poisoning and healthy control individuals. Elevated levels of the α 1-m, β 2-m, TRF, IgG, and NAG proteins were observed in both acute and chronic Hg-exposed patients, suggesting that urine samples may be a simple and inexpensive way to test for Hg exposure.

In addition to studying the adverse effects of metal exposure, it is important to try to develop methods of exposure prevention. For example, Huang's group attempted to develop a correlation model between Fe exposure in coal mines and Coal Workers' Pneumoconiosis (CWP). Fe is an essential trace metal that is stored in Fe-containing proteins, such as ferritin and transferrin, where it is tightly bound and unavailable to cause oxidative damage [17–19]. Bio-available iron (BAI), such as Fe associated with citrate and ATP, has been shown to produce oxidants in a pH-dependent manner [20]. Some coal dusts contain BAI, and have been shown to activate the stress-inducible AP-1 and NFAT transcription factors, while coal dust lacking BAI had no effect on either

transcription factor [21]. Previously, BAI content in Pennsylvanian coal was shown to be much higher than the BAI content in coal dust originating from Utah [22]. Huang posited that BAI content could be used as a predictor of coal toxicity. The BAI content of coal from Utah and Pennsylvania was calculated and compared to epidemiological studies reporting the incidence of CWP. A significant correlation was found between BAI levels and CWP incidence, suggesting that BAI levels may be used to predict the toxicity of coal before mining begins.

A second example of an *in vitro* predictive model for metal-induced disease was introduced by Hockertz *et al.* Primary mast cells from human lung tissue and foreskin specimens were exposed to wear particle antigens including chromium–cobalt–molybdenum alloy (CrCoMo), a titanium alloy (TiAL6V4), or ultra-high molecular weight polyethylene (UMHWPE), all of which can be released into the human body by the degradation of implanted osseoprostheses [23]. Histamine release, indicative of an allergic reaction, was observed when the antigens were added to serum-containing media and applied to primary mast cells. If a patient’s IgE was present, histamine release increased, particularly for the CrCoMo antigen. This *in vitro* model could be useful in preventing potentially dangerous allergic reactions in patients with osseoprostheses and reduce the need for the administration of immunosuppressants.

In some cases, a single biomarker candidate may be difficult to obtain and a profiling-based approach may be useful in identifying a molecular fingerprint for toxicant exposure. Boyd *et al.* presented an example of a high throughput strategy utilizing an automated robotic workstation (COPAS BIOSORT) to dispense and measure the length of *Caenorhabditis elegans* worms. Toxicological endpoints were examined using a

fluorescent dye that only penetrates deceased worms. An imaging workstation was used to track the motion of the live worms and acquire multidimensional image analysis data. This type of high throughput method can be used to examine the properties of potential neurological and developmental toxicants and detect subtle changes in gene or protein expression that may be undetectable by conventional methods.

Balancing act: The role of reactive oxygen species (ROS) and antioxidants

Many transition metals have been shown to produce elevated amounts of damaging ROS [24–28]. Disturbing the redox balance of a system leads to an increase in DNA damage, DNA-protein crosslink formation, lipid peroxidation, apoptosis, cellular toxicity, and/or the inappropriate activation of cellular signaling pathways [29–35]. Since the mechanisms of ROS action are not well understood, many conference participants focused their studies on the elucidation of these mechanisms. For example, Cr(VI) is a transition metal that is known for its ability to produce increased quantities of ROS in cellular systems, particularly $\cdot\text{OH}$ [36, 37]. Previous studies have focused on the effects of soluble Cr(VI), since solubility is thought to be an important factor in its ability to contribute to carcinogenesis [38]. However, water insoluble (or particulate) Cr(VI) has also been shown to be genotoxic and cytotoxic [24, 39–41]. Leonard *et al.* examined the capability of insoluble Cr(VI) in the form of PbCrO_4 to produce ROS using electron spin resonance (ESR) spectroscopy and the comet assay. Their results showed that insoluble Cr(VI) was capable of producing oxidative stress in a similar pattern to that of soluble Cr(VI).

Additional studies examining the effects of PbCrO_4 in the human lung cell line WHTBF-6, were presented by Wise *et al.* PbCrO_4 was shown to enter the lung cells and exert clastogenic effects in a dose-dependent manner, although dicentric chromosome formation was not observed. In order to exclude Pb ions as the culprits of clastogenicity, HTBF-6 cells were also exposed to Pb-glutamate, which yielded no clastogenesis. However, Na_2CrO_4 treatment also proved to be clastogenic in the lung cells, suggesting that the CrO_4^{2-} ions were the cause of clastogenicity. In addition, the antioxidant vitamin C prevented the uptake of CrO_4^{2-} ions and exhibited only a slight effect on Pb levels. The cytotoxicity profiles of WHTBF-6 cells exposed to CrO_4^{2-} ions and PbCrO_4 were comparable. Both PbCrO_4 and Na_2CrO_4 induced cell cycle arrest and inhibited growth, whereas Pb ions alone induced only mitotic arrest.

ROS production often leads to cell death through cytotoxicity and/or apoptosis, prompting several groups to examine the benefits of inducing ROS in a cellular system through metal treatment. Farmer *et al.* used synthetic melanins derived from dihydroxy indole (DHI) to study the speciation, metal-binding affinity, and redox reactivity of those synthetic melanins. Several metals bound the DHI-melanin, increasing redox cycling and the production of $\cdot\text{OH}$ and $\text{O}_2^{\cdot-}$ surprisingly. Indium(III) ions alone and lipophillic metallodithiocarbamate exhibited an unusual amount of toxicity towards melanoma cells in culture. The dithiocarbamate disulfide disulfiram (DSF), which is traditionally prescribed as an alcohol-abuse deterrent, also showed strong Cu-dependent activity towards melanoma cells. Cen's group confirmed that DSF exhibited a Cu-dependent toxicity towards melanoma cells, whereas nontransformed melanocytes were only slightly affected. Upon DSF treatment, melanoma cell survival decreased to less than

10% as the intracellular Cu concentrations increased. The addition of Cu chelators blocked the DSF-induced cell toxicity and Cu uptake by melanoma cells. Formation of a Cu(deDTC)₂ complex was proposed to be the likely active agent. This response is unique to Cu(II) and was not observed with Fe(II), Mn(III), or Zn(II), suggesting that local administration of Cu and DSF or DHI may be useful in the treatment of melanoma.

Exposure to metal-containing particulates can also increase ROS production in cellular systems. For example, diesel exhaust particles (DEP) have been shown to induce DNA adducts and increase inflammation in the respiratory tract [42–46]. DEP contents include polycyclic-aromatic hydrocarbons (PAHs), redox active semi-quinones, and trace heavy metals [47, 48]. Park *et al.* compared ROS production of the DEP standard reference material 2975 (SRM 2975) and DEP collected from the air in Korea as measured by thiobarbituric acid – reactive substances of deoxyribose (TBARS). Differences in transition metal composition were observed between samples; DEP from Korea contained more Fe, less Cu, and less Zn than SRM 2975. TBARS measurements of the water soluble fraction of SRM 2975 showed a broad absorption in the visible range, which was absent in the DEP from Korea. Although SRM 2975 increased the amount of TBARS fluorescence, H₂O₂ generated more TBARS in the DEP from Korea, probably due to the increase in Fe composition. These results support a role for Fe in DEP-induced H₂O₂ toxicity, which may be the underlying cause of the adverse health effects of DEP.

Some substances seem to exert both pro-oxidant and antioxidant effects. Recently, many epidemiological and experimental studies have suggested that drinking green or black tea may protect against cancer [49, 50]. Sinha *et al.* presented evidence suggesting that tea extracts, in particular the polyphenolic components, exert their

chemopreventative effects by acting as antioxidants. They proposed that tea extracts can prevent DNA damage caused by sodium arsenite in V-79 Chinese hamster lung fibroblasts. However, Azmi *et al.* presented evidence that the presence of at least one of those polyphenolic components, (-) epigallocatechin-3-gallate (EGCG), can induce $\cdot\text{OH}/\text{O}_2^{\cdot-}$ formation and increase the rate of DNA oxidation in the presence of Cu ions. The results of Azmi's group suggest that EGCG's apoptosis-inducing pro-oxidant properties may be more important than its preventative antioxidant properties. These studies are also supported by the findings of Qanungo *et al.* and Zykova *et al.* which suggest that tea polyphenols may serve as either an antioxidant or an oxidant, depending on the physiological environment [51–53].

Ascorbate/ascorbic acid/vitamin C is another compound that has been shown to exhibit both pro-oxidant and antioxidant effects [54–58]. Huq and Hussain presented evidence suggesting that exposure to Cd acetates increases DNA damage in the presence of ascorbate. The mechanism occurs via the formation of a 1:1 covalent Cd(II)/ascorbate adduct, causing a molecular activation of the transition metal. This group proposed that the deprotonated ascorbate ions bind to $^3\text{O}_2$ producing the volatile ROS species, singlet molecular oxygen ($^1\text{O}_2$).

Ironically, some transition metals may serve as antioxidants and prevent oxidative cellular damage. Aravind and Prasad presented evidence that Zn may be able to prevent Cd toxicity. *Ceratophyllum demersum* L. plants were treated with different concentrations of CdCl₂ and/or ZnCl₂. The production of $\cdot\text{OH}$, an oxidative shift in GSH levels, loss of carbonic anhydrase conformation, and DNA fragmentation were observed with CdCl₂ treatment. Upon treatment with both CdCl₂ and ZnCl₂, the presence of Zn

inhibited all of the aforementioned markers of oxidative damage, suggesting that Zn may be able to prevent the cytotoxic effects induced by Cd.

Rhenium is a second transition metal that exhibits low ROS-induced toxicity. Thus, cluster rhenium compounds (CRCs) are thought to be a potential drug delivery agent. CRCs possess lower toxic effects than other metals currently used as anti-tumour agents, including palladium, platinum, and cisplatin [59]. Shtemenko and Shtemenko examined the mechanisms of lipid-CRC interactions in solution and during the formation of liposomes. They had previously shown that CRCs bind to carboxylic acids, amino acids, or adamantanic acids, and subsequently interact with the surface of red blood cells to stabilize the membrane in response to free radicals [60]. Administration of CRC1-dichlorotetra- μ -(i-butirato) dirhenium(III) or CRC2-tetrachlorodi- μ -(γ -aminobutirato) dirhenium(III) chloride in models of hemolytic anemia led to an increase in hemoglobin and resistance of erythrocytes to oxidative stress, extending the life of hemolytic animals. In addition, CRC1 or CRC2 administration increased reduced glutathione pools, glutathione reductase expression, and glutathione peroxidase (GPx) expression in the tissues of animals with hemolytic anaemia. Thus, CRCs demonstrate antioxidant and antiradical properties *in vivo* and may be useful in the treatment of hemolytic anaemia.

Cellular systems have developed a wide range of antioxidant mechanisms to combat the dangerous accumulation of ROS. Examples include the increased expression of ROS detoxification enzymes, such as catalase and superoxide dismutase (SOD), and the maintenance of pools of small molecular ROS scavengers, such as N-acetyl-cysteine (NAC) and reduced glutathione (GSH). These ROS scavenging molecules react with excess ROS, thus minimizing cellular damage. Glutathione (GSH), although widely

known for its antioxidant activity, is also involved in Se metabolism and bioactivity [61]. Shen *et al.* presented evidence that Se caused a dose-dependent onset of mitochondrial permeability transition (MPT) in the presence of GSH, which could be inhibited by the MPT inhibitor cyclosporin A. An SOD mimic, Mn[III]tetrakis [4-benzoic acid] porphyrin, has been previously shown to prevent MPT caused by xanthine (X)–xanthine oxidase (XO) [62]. Surprisingly, SOD had no effect on MPT and was unable to prevent the reduction of cytochrome *c* induced by selenite-GSH (Se-GSH) formation. Although others have observed an increase in $O_2^{\bullet-}$ formation during Se-GSH generation, Shen's group measured $O_2^{\bullet-}$ via the lucigenin assay and found no significant increase in $O_2^{\bullet-}$ when compared to $O_2^{\bullet-}$ production by the X–XO pathway [63]. These results suggest that $O_2^{\bullet-}$ is not responsible for Se-GSH-induced MPT in this system.

Resveratrol is a polyphenolic antioxidant that is synthesized by many plant species and has been shown to inhibit the growth of cultured cancer cells [64]. Xia *et al.* presented evidence that ROS-induced activation of the PI3K/Akt survival pathway can be inhibited by the presence of resveratrol in prostate cancer cells. Resveratrol also inhibited the expression of proteins involved in angiogenesis, such as HIF-1 α and VEGF, in a dose-dependent manner. Xia's results suggest that resveratrol may be able to inhibit prostate cancer progression and angiogenesis through the inhibition of these pathways.

DNA damage/lesions and mitotic effects

In addition to producing ROS, many metals have been shown to directly modify and/or damage DNA by forming DNA adducts, facilitating DNA protein crosslink formation, or inducing chromosomal breaks [33, 44, 65, 66]. Many researchers at the

conference presented work reflecting efforts to identify the mechanisms of metal-induced DNA damage. For example, O'Brien *et al.* examined the role of O₂ in Cr(VI)-induced DNA lesions and found that hypoxic conditions did not alter Cr(VI) reduction by ascorbic acid. However, both Cr(VI)-DNA binding and the occurrence of polymerase arresting lesions (PALs) decreased under hypoxic conditions by 70% and 50–90%, respectively. These results suggest that O₂ can help facilitate the formation of Cr(VI)-DNA complexes.

A common oxidative DNA modification, the 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxo-dG) lesion, can lead to the misincorporation of extraneous nucleotides during DNA replication [67, 68]. The 8-oxo-dG lesions are prone to further oxidation by many transition metals, including Cr(V) and Iridium (IV). Further oxidation of 8-oxo-dG leads to the formation of guanidinohydantoin (Gh) and spiroiminodihydantoin (Sp) lesions, subsequently causing G→T and G→C transversions [69, 70]. Gh and Sp lesions are thought to enhance base misincorporation and the polymerase blocking effects of the 8-oxo-dG lesion [71, 72]. Certain base excision repair (BER) enzymes recognize 8-oxo-dG lesions, but not Gh or Sp lesions [73, 74]. Hailer *et al.* showed that the mammalian BER glycosylases NEIL1 and NEIL2 both recognize and cleave Gh and Sp lesions in single-stranded DNA [75]. These results demonstrate that an alternative pathway is activated in response to the formation of Gh and Sp DNA lesions. The 8-oxo-dG lesion also affects transcription factor binding [76, 77]. Previous studies by Sugden's group reported that binding activity of the NF-κB p50 subunit increases if the G₁ nucleotide of the NF-κB consensus binding sequence (5'-dAGTTGA G₁G₂G₃G₄ACTTTCCCAGCC-3') is replaced with an 8-oxo-dG modified base [77]. In

contrast, replacing the G₃ nucleotide with an 8-oxo-dG decreases p50 binding activity. Sugden *et al.* found that p50 binding activity to the modified NF- κ B consensus sequences correlated with p50 *in vitro* transcription activity, providing a direct link between the regulation of gene transcription and ROS formation.

In addition to inducing DNA lesions, metals can interfere with cellular processes such as mitosis. Kligerman *et al.* presented evidence that arsenicals induced c-type anaphases consisting of small, condensed chromosomes – an effect usually seen with spindle poisons [78]. They proceeded to investigate the effects of several different modified arsenicals on mitosis and found that monomethylarsenous acid (MMA^{III}) exhibited the most potent mitotic interference. When tubulin was directly exposed to MMA^{III}, an increase in the number of mitotic indices (MIs) was observed. None of the arsenicals with a valence state of V showed an obvious effect on tubulin polymerization, whereas all arsenicals with a valence state of III inhibited tubulin polymerization. Taken together, these results indicate that As(III) and its metabolites can interfere with cell division.

Activation of DNA repair enzymes is often observed in response to metal-induced DNA damage [79–81]. Lee *et al.* examined the effects of DNA repair gene polymorphisms on the number of Cr(VI)-induced strand breaks in human white blood cells. Several DNA repair genes with known genetic polymorphisms were examined, including glutathione S transferase M1 (GSTM1), glutathione S transferase T1 (GSTT1), NADPH quinone oxidoreductase 1 (NQO1), X-ray repair cross complementation factor 1 (XRCC1), and the 8-oxo-7,8-dihydroguanine (8-oxo-G) DNA glycosylase (OGG1). White blood cells homozygous for the OGG1 Cys³²⁶ polymorphism exhibited an

increased number of DNA strand breaks when compared to the OGG1 Ser³²⁶/Ser³²⁶ and OGG1 Ser³²⁶/Cys³²⁶ polymorphisms. White blood cells homozygous for the OGG1 Cys³²⁶ variation also exhibited an increase in the ratio of oxidative DNA damage to plasma antioxidant capacity. These results suggest that OGG1 Cys³²⁶ homozygous individuals may be defective in the repair of DNA adducts during oxidative stress conditions, possibly due to the oxidation of these residues.

Metal-induced cellular signaling

Although each metal activates its own unique set of signaling events, many metals also activate general ROS-mediated stress response pathways. DNA microarray technology has recently been used to examine the global effects of metals in several model systems. For example, Jin *et al.* examined the global transcriptional response of the yeast *Sacchomyces cerevisiae* to Zn, Cd, Hg, Cu, silver (Ag), Cr, or arsenic (As) exposure. Approximately 25% of the yeast genes were affected by at least one metal (798 upregulated genes and 774 downregulated genes). Most of the metals upregulated genes involved in the oxidative damage process, sulfur assimilation, sulfur metabolism, and GSH biosynthesis. Genes encoding biomolecular transporters of sugar and lipid metabolism were downregulated. Approximately 10% of the total number of *S. cerevisiae* genes were affected by As treatment – a 2–5-fold increase in the number of genes affected by any other metal examined. Additional classes of genes induced by As treatment included double-stranded break (DSB) repair and DNA replication genes.

Other examples of the use of microarray analysis in toxicology studies included Song and Freedman's examination of the global transcriptional effects of Cu treatment on

a human hepatoma cell line, Hep2G. After 4 h of Cu exposure, 42 genes were upregulated and 24 were downregulated. The upregulated genes included metallothionein IIA (MT2A), myosin heavy polypeptide (MYH8), CYP1A1, IL-1 receptor alpha (IL11RA), heat shock protein A1A (HSPA1A), and heme oxygenase 1 (HO-1). Downregulated genes included the HT017 protein, dynamin 1-like (DNM1L), DEAD box polypeptide 18 (DDX18), and GST theta 2 (GSTT2). RT-PCR confirmed the upregulation of HSPA1A, CYP1A1, and HO-1 with peak induction levels at 12, 24, and 8 hours respectively.

Many of the global effects of metals on transcription are thought to be controlled by the interactions between metal response elements (MREs) and MRE binding transcription factors such as MTF-1 [82]. Since PKC inhibitors can abrogate MT induction, it is thought that some metals induce signaling cascades through PKC activation, subsequent alteration of MTF-1 phosphorylation, and activation of MT transcription [83]. Craft and Freedman examined the role of p53 activation in MT induction by Hg and Cd and found MT transcription was reduced in p53 $-/-$ cells when compared to wild-type p53 cells. These results suggest that p53 may be part of a general mechanism in the cellular response to both Hg and Cd exposure.

Arsenic signaling

After a metal ion is transported across the cytoplasmic membrane, its transition state is often altered by cellular reducing agents. Reduction of some metals to a lower transition state, such as As(V) \rightarrow As(III), often results in an increase in toxicity; thus it is

beneficial to examine the metal's cellular point of entry. The ArsATPase is a catalytic subunit of the *Escherichia coli* ArsAB pump that translocates As(III) across the cellular membrane, conferring resistance to arsenicals and antimonials [84, 85]. There are two halves of the Ars ATPase, A1 and A2, each consisting of a nucleotide binding domain (NBD) and a metal binding domain (MBD). The NBD and MBD are connected by two amino acid linkers which serve as a signal transduction domain [85, 86]. Using site-directed mutagenesis, Bhattacharjee and Rosen altered residues of the signal transduction domain and found that the D142A and P145A mutations caused a loss of As(III) resistance. These results indicate that this domain is important in the signaling events between the catalytic and allosteric sites of the Ars ATPase. The aquaglyceroporin (AQP) family is responsible for the uptake of As(III) and Sb(III) in humans [87, 88]. Mukhopadhyay *et al.* reported that overexpression of human AQP9 in the chronic myelogenous leukemia cell line K562 increased uptake of the As(III)-based drug Trisenox [89]. Since AQP9 overexpression in K562 cells exhibited hypersensitivity to Trisenox, it is possible that pharmacological alteration of AQP9 expression could boost Trisenox effectiveness in cancer patients.

As(III) induces oxidative stress and delays cell cycle reentry into the G₁ phase by an unknown signaling mechanism [90, 91]. Chen *et al.* presented evidence that As(III) induces ubiquitination and subsequent degradation of the Cdc25C protein, a protein which is thought to cause the exit of cells from the G₂/M phase [92]. These effects were not abrogated by treatment with antioxidants. Furthermore, immunoprecipitation showed that Cdc25C associates with the Fzr/Cdh1 recognition subunit of the mitotic APC ubiquitin ligase complex. siRNA silencing of Fzr/Cdh1 expression protected Cdc25C

from degradation and decreased ubiquitin conjugating activity. Chen's group concluded that As(III) induces the redox-independent ubiquitination and degradation of Cdc25C through its association with Fzr/Cdh1.

As(III) exposure also induces remodelling of the cytoskeleton [93]. Qian *et al.* demonstrated that As(III) activated Cdc42 and NADPH oxidase. Activated NADPH oxidase produced $O_2^{\bullet-}$ and induced actin filament reorganization in SVEC4-10 cells [94]. Overexpression of a dominant negative Cdc42 or treatment with the actin filament stabilization agent jasplakinolide inhibited actin filament reorganization and prevented NADPH oxidase activation. Qian's group concluded that As(III) activates NADPH oxidase through Cdc42 and leads to actin filament reorganization and the formation of lamellipodia and filopodia. These effects could possibly lead to increased cell migration and/or metastasis of As(III)-induced tumors.

Paradoxically, As also exhibits apoptotic-inducing effects. Ivanov and Hei presented evidence that human melanoma cells exposed to combinations of low concentrations of As (2–10 μ M) and EGFR, PI3K/Akt, or MEK/Erk inhibitors effectively induced apoptosis via the TRAIL and TNF α pathways in spite of low Fas levels [95]. The combination of As and synthetic small molecule inhibitors could potentially be used to sensitize melanoma cells to cytotoxic drugs, enabling the development of more effective chemotherapy regimens.

Lemarie *et al.* further examined the apoptotic pathway induced by As and found that As₂O₃ prevented monocyte adhesion and expression of the macrophage phenotypical markers transferrin receptor CD71 and integrin CD11c. As₂O₃ induced monocyte apoptosis, increased caspase 8 and caspase 3 expression, decreased cFLIP and XIAP

expression, decreased NF κ B binding activity, and exhibited a loss of mitochondrial membrane potential. Lemarie's group suggested that As₂O₃ may induce apoptosis by inhibiting NF κ B activity and subsequently decreasing the expression of the cFLIP and XIAP pro-survival proteins, both of which are transcriptionally controlled by NF κ B. Taken together, these studies may provide insight into the signaling pathways responsible for As's ability to induce apoptotic effects as well as carcinogenic effects.

Chromium signaling

Cr(VI) has been known to produce ROS, induce DNA damage, and subsequently activate the ATM, p53, and Chk2 proteins [96, 97]. It is known that ATM is activated specifically in response to DSBs, however no Cr-induced DSBs have been reported [98–101]. Ceryak *et al.* exposed normal human dermal fibroblasts to Cr(VI) or the radiomimetic agent neocarzinostatin (NCS) and analysed the occurrence of DSBs, the phosphorylation status of histone H2A.X (γ -H2AX), and the formation of γ -H2AX nuclear foci. Their evidence suggested that DSBs were formed in response to Cr(VI) exposure in an S phase-dependent manner, while NCS exposure exhibited an equal distribution of DSB formation throughout all phases of the cell cycle. Additionally, Cr(VI) exposure showed γ -H2AX foci formation in PCNA positive cells; an effect that was absent in the NCS-exposed control cells and decreased in ATM^{-/-} cells.

Xie *et al.* independently showed that PbCrO₄ induced DSBs in a dose-dependent manner, resulting in the activation of ATM in human lung cells. γ -H2AX is rapidly phosphorylated on Ser¹³⁹ when DSBs are induced by ionizing radiation [102]. Xie's group also showed that PbCrO₄ induced a concentration-dependent phosphorylation of γ -

H2AX and subsequent foci formation. Taken together, these results demonstrate a general mechanism for Cr(VI)-induced carcinogenesis involving the induction of S phase-dependent DSBs and marked by γ -H2AX foci formation.

Xu presented additional evidence that Cr(VI) exposure activates ATM/ATR, phosphorylates the structural maintenance chromosome 1 (SMC1) protein, and activates caffeine sensitive S phase cell cycle arrest in a dose-dependent manner. Surprisingly, Cr(VI)-induced S phase cell cycle arrest was shown to be independent of ATM at high concentrations. Xu proceeded to examine alternate pathways responsible for Cr(VI)-induced S phase cell cycle arrest and found that Rad17, which is required for the release of active ATR, bound the site of Cr(VI)-induced DNA damage. A non-functional Rad17 showed impaired S phase arrest in response to Cr(VI) exposure, suggesting that a low dose of Cr(VI) activates an ATM-dependent pathway, whereas a high dose of Cr(VI) activates an ATR-dependent pathway.

Cadmium signaling

The activation of transcription factors by metals is one of the most commonly studied effects of metal-induced cellular signaling. However, recent interest has been expressed in alternative targets of metal signaling, such as the translational machinery pathways. Aberrant regulation of the expression of the translational control proteins eIF3 and eEF1 δ has been shown to be responsible for Cd(II)-induced transformation and tumorigenesis [103, 104]. Orthumpangat *et al.* presented evidence that a third translational control protein may also affect the cellular response to metals. Cell lines exposed to Cd(II) exhibited a decrease in eIF4E protein expression. siRNA silencing of

eIF4E induced cell death, whereas eIF4E overexpression resulted in cell survival after exposure to an otherwise lethal dose of Cd(II). Cd(II) exposure also activated the ubiquitin pathway, resulting in the degradation of eIF4E and a subsequent decrease in cyclin D1 expression.

The expression of other members of the translational machinery family were altered upon exposure to Cd(II). One such protein, eEF1A2 is a cellular proto-oncogene which is overexpressed in many cancer cell lines and tumors. Orthumpangat's group reported that eEF1A2 expression was increased in response to Cd(II), while overexpression of eEF1A2 conferred resistance to Cd(II)-induced apoptosis. These results confirm that the translational machinery is a potential mechanism for Cd(II)-induced carcinogenesis.

Hep3B human hepatocarcinoma cells have been shown to undergo Cd-induced apoptosis in a caspase-independent manner [105]. The mechanism is thought to involve the nuclear translocation of endonuclease G (endoG) and the apoptosis-inducing factor (AIF), both of which are mitochondrial apoptogenic proteins. Lemarie *et al.* showed that the release of endoG and AIF in response to Cd exposure was preceded by an increase in cytoplasmic Ca^{2+} and a loss of mitochondrial membrane potential. Bapta-AM, a Ca^{2+} chelator, blocked these events and prevented apoptosis. Inhibition of ROS production by the mitochondrial inhibitors ruthenium red, rotenone, and diphenyleneiodonium prevented the loss of mitochondrial membrane potential. Bapta-AM and diphenyleneiodonium also blocked expression of the NF κ B-regulated anti-apoptotic protein bcl-x(L) in Cd exposed cells. Lemarie's group concluded that Cd induces apoptosis in Hep3B cells through Ca^{2+} release and ROS-induced impairment of the

mitochondria. These results are in agreement with the hypothesis that Cd-induced apoptosis occurs through the release of endoG and AIF.

Cobalt and copper signaling

Exposure to the hard metal dust tungsten carbide–cobalt mixture (WC–Co) directly correlates with an increased risk of cancer in exposed workers [106]. Lombaert *et al.* demonstrated that both cobalt (Co) and WC–Co induced apoptosis of peripheral blood mononucleated cells (PBMCs). WC–Co apoptosis occurred at a higher rate than Co-induced apoptosis and was dependent on caspase 9 activation, whereas Co-induced apoptosis depended on the activation of both caspases 8 and 9. These results demonstrate that although WC–Co and Co can produce similar cellular effects, the signaling mechanisms may be different.

Although Cu is a trace element necessary for the function of enzymes such as SOD, it can also produce ROS, DNA damage, and other carcinogenic effects when present in excess. Cherian and Ostrakhovitch examined the effects of Cu exposure on the p53 tumour suppressor signaling pathways in the breast cancer epithelial cell lines MDA-MB-231 and MCF7. p53 mutant MDA-MB-231 cells are resistant to metal toxicity, while p53 wild-type MCF7 cells are sensitive to metal-induced apoptosis [107, 108]. ROS production was not observed in MDA-MB-231 cells exposed to Cu; however, an increase in Akt phosphorylation, Akt nuclear translocation, Cyclin D1 expression, and cell cycle progression were still apparent. In contrast, ROS production in MCF7 cells increased in response to Cu exposure. Increases in p53 expression, p21 expression, G₁ phase arrest, and apoptosis in MCF7 cells were also observed. Additionally, Cu-exposed MCF7 cells

exhibited a loss of mitochondrial membrane potential and lacked Akt activation and translocation. Suppression of p53 in MCF7 cells with pifithrin or E6 protein decreased p53 phosphorylation and increased Akt phosphorylation, suggesting that p53 is involved in the response to Cu-induced ROS formation [109].

Nickel signaling

Insoluble nickel (Ni) compounds, such as Ni₃S₂, green NiO, black NiO, and Ni refinery dust, have been shown to exert carcinogenic effects in a variety of animal experiments [110]. Landolph *et al.* showed that Ni₃S₂, green NiO, and black NiO are phagocytosed by C3H/10T1/2 mouse embryo cells. Cytotoxicity, chromosomal aberrations, and morphological transformation were observed upon Ni exposure. The extent of phagocytosis was shown to correlate with each Ni compound's ability to induce transformation. RAP-PCR differential display experiments showed 130 genes that were differentially expressed between the Ni-transformed and nontransformed cell lines. Increased expression of the *ect-2*, *calnexin*, and *wdr1* genes in transformed cells were confirmed by Western blot. Respectively, these genes encode a Rho GDP/GTP exchange factor that modulates microtubule assembly, a molecular chaperone, and a stress-inducible protein. Decreased expression of the vitamin D receptor interacting protein/thyroid hormone activating protein 80 (DRIP/TRAP-80) and two other novel genes was also observed in transformed cells. Landolph's group concluded that Ni ions generate ROS, mutate and activate proto-oncogenes and/or tumour suppressor genes, and also induce chromosomal aberrations.

Paradoxically, Ni can upregulate the expression of some genes while downregulating others, possibly through the post-translational modification of histones. Ni suppresses the acetylation of core histones H3 and H4 and induces *de novo* DNA methylation, leading to gene silencing [34,111]. Costa's group found that water-soluble Ni salts could inhibit the acetylation of the core histones H2A, H2B, H3, and H4. Ni exposure also induced methylation of Lys 9 in histone H3 and increased the ubiquitination of histones H2A and H2B. Golebiowski and Kasprzak also examined the acetylation status of the N-terminal tails of the core histones in Ni-exposed human and rodent cells exposed to Ni(II). Histone H2B exhibited the least acetylation, followed by histones H4, H3, and H2A in human airway epithelial cells and normal rat kidney cells. However, these effects were not seen in Chinese hamster ovary (CHO) cells, suggesting that the decrease in acetylation may be cell-type dependent. Ni(II) has been shown to cleave the -SHHKAKGK motif of the C-terminal tail of histone H2A *in vitro* and in cell culture [112, 113]. Karaczyn *et al.* examined the effects of Ni(II) on other histones and showed that histone H2B abundance increases with Ni(II) treatment over time. Mass spectrometry and amino acid sequencing analysis confirmed that the H2B immunoreactive bands were variants of histone H2B. The larger band represented a truncated histone H2B lacking 16 amino acids from the N-terminal tail. The smaller band represented a histone H2B variant lacking both the 16 N-terminal amino acids and an additional nine amino acids from the C-terminal tail. Post-translational modifications of the smaller band included two acetylated Lys residues. Similar, but weaker effects on histone H2B were seen with Co(II), but no effects were seen with Cu(II), Cd(II), or Zn(II). Since the terminal tails of histones are thought to play key roles in structuring

chromatin and regulating gene expression, the loss of these regions may be involved in the mechanisms relating to Ni(II)-induced carcinogenesis. Taken together, these results suggest that histone modification may play a major role in the mechanism of Ni-induced gene activation and/or silencing.

In addition to modifying core histones, Ni-exposure also activates the HIF-1 α transcription factor via the depletion of intracellular Fe and the subsequent inhibition of the Fe-dependent enzyme proline hydroxylase, which leads to the stabilization of HIF-1 α [114]. Huang *et al.* examined the effects of Ni₃S₂ or NiCl₂ exposure on other transcription factors and found that Ni ions increased ROS production and activated NFAT. NFAT activation was inhibited by catalase, NAC, and desferoxamine (DFO), but not by SOD or sodium formate. Ni activation of the HIF-1 α , PI3K, Akt, and p70S6K was abrogated by PI3K inhibitors. Overexpression of Akt and PI3K dominant negative (DN) mutants also inhibited HIF-1 α activation and Cap43 expression, while exposure to rapamycin, a p70S6K inhibitor, had no effect. In conclusion, Huang's group suggested that Ni generates H₂O₂ which in turn activates NFAT. HIF-1 α and Cap43 are also activated by Ni via a PI3K/Akt-dependent pathway, independently of p70S6K activity.

Animal studies

Although animal studies provide valuable information concerning the toxic effects and signaling pathways of environmental contaminants, adequate animal models do not exist for some metals due in part to their roles as co-carcinogens. Thus, it is difficult to assess the effects of the metal itself because a known carcinogenic compound must be administered simultaneously in order for a tumour to develop. For example,

Burns *et al.* presented evidence that hairless mice (Skh1strain) exposed to ultra-violet radiation (UVR) and Cr- or As-contaminated drinking water elevated the number of tumors produced by UVR to 8.3- and 4.6-fold, respectively. These data confirm that metals can act as hazardous co-carcinogens even at low levels of exposure.

Most metal toxicity studies have been conducted on adult animals, yet few have focused on the effects of metal exposure during the pubescent period. Chatterjee *et al.* examined Sprague–Dawley rats exposed to intravenous-infused CdCl₂ 40–100 days after birth. Histopathological examination of the mammary tissue showed that Cd(II) exposure slightly suppressed apoptosis while DNA strand breaks increased by 61%. At low doses, no change in histopathology of the mammary tissue was observed. However, at high doses, exposed rats exhibited intraductal proliferations. In addition, MT expression was elevated 60–86% in Cd(II)-treated animals in a dose-dependent manner. These results indicate that Cd(II) exposure may lead to an increased incidence of cancer in pubescent animals.

Additionally, Chatterjee *et al.* examined the chemopreventive effects of vanadium (V) supplementation. Rats were exposed to diethylnitrosamine (DEN) and phenobarbital to induce hepatocarcinogenesis, and subsequently fed V-supplemented chow for 20 weeks. V supplementation reduced the nodular incidence, total number of tumors, and multiplicity of tumors, and decreased metallothionein expression, BrdU labelling index (a marker of cell proliferation), and iNOS expression. In addition, V supplementation increased p53 immunoreactivity and the apoptotic labeling index. These results suggest that V may play a significant role in controlling cellular activities during chemical-induced hepatocarcinogenesis.

Depleted uranium (DU) is a radioactive heavy metal used to manufacture military munitions. United States military personnel wounded by DU shrapnel may have a higher risk of cancer incidence, due to DU's genotoxic and mutagenic effects [115, 116]. DU can also cause radiation-specific cellular damage and transformation of human osteoblast cells [117, 118]. In order to simulate the effects of a DU-containing shrapnel wound, Miller *et al.* implanted non-tumourigenic immortalized cells into syngenic DBA/2 mice with or without DU implantation pellets. Mice implanted with DU pellets showed an 80% incidence of tumorigenesis, whereas control mice exhibited a 10% incidence of leukemia, suggesting that DU can significantly enhance tumour incidence.

Chromium(III) Picolinate ($\text{Cr}(\text{Pic})_3$) is a dietary supplement associated with oxidative damage, DNA damage, antioxidant enzyme depletion, and renal/liver dysfunction in rats [119, 120]. In cell culture, $\text{Cr}(\text{Pic})_3$ has been shown to cause mutations and DNA fragmentation [121]. Rasco *et al.* examined the prenatal effects of dietary supplementation of $\text{Cr}(\text{Pic})_3$, CrCl_3 , or picolinic acid in pregnant female mice. The fetuses birthed from the $\text{Cr}(\text{Pic})_3$ -fed females showed an increase in bifurcated cervical arches, delays in righting reflex, delays in hind limb grasp, and deficiencies in motor skills. These results demonstrate that dietary supplementation of $\text{Cr}(\text{Pic})_3$ exerts negative effects on the developing nervous system of mammalian fetuses.

Barchowsky *et al.* developed an animal model to examine the effects of As(III) on angiogenesis and the expression of tissue remodelling genes in cardiac tissue. Male mice were exposed to As(III)-contaminated drinking water at low to moderately high concentrations for up to 20 weeks. Enhanced vascularization of Matrigel implants was observed after 5 weeks of As(III) exposure. RT-PCR showed a dose and time-dependent

induction of VEGF, VEGF receptors, plasminogen activator inhibitor-1, endothelin-1, and matrix metalloproteinase-9 in cardiac tissue. Barchowsky's group concluded that the effects of chronic As(III) exposure on the cardiovascular tissue varies with dose and length of exposure.

Summary

The research presented at the 3rd Conference on Molecular Mechanisms of Metal Toxicity and Carcinogenesis provided new insights into the mechanisms of ROS production, the mechanisms of metal signaling, and the development of animal exposure models. However, the following areas of metal research still need further exploration. First, there is a need for the development of new human biomarkers for metal exposure. Without definitive and specific biomarkers, it is very difficult to assess the extent of metal exposure in humans, and thus, difficult to treat the resulting ailments. Second, since most metals cause some form of ROS-induced cellular stress, the elucidation of the mechanisms of ROS production may provide information concerning the general mechanisms of metal-induced carcinogenesis. Third, defining the mechanisms of metal-induced DNA damage may provide new insights into the signaling events involved in DNA damage repair pathways. Fourth, a better understanding of the complex signaling networks that lead to the adverse effects of metals could lead to the identification of superior and more specific novel therapeutic agents. Finally, better animal models for metal exposure are necessary in order to thoroughly evaluate the toxic and carcinogenic effects of metals in a model system. Through the use of current technology and the

implementation of new experimental strategies, metal researchers can address these concerns.

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SECTION IV

**As(III) transcriptionally activates the *gadd45a* gene via the
formation of H₂O₂**

ABSTRACT:

Arsenic is a ubiquitous environmental contaminant associated with an increased risk of human cancers of the skin, lung, bladder, and prostate. Intriguingly, it is also used to treat certain types of leukemia. It has recently been suggested that these paradoxical effects may be mediated by arsenic's ability to simultaneously activate DNA damage, apoptotic, and transformation pathways. Here, we investigate the effects of arsenic exposure on the induction of the growth arrest and DNA damage protein 45 α (GADD45 α), which is thought to play roles in apoptosis, DNA damage response, and cell cycle arrest. We found that arsenic transcriptionally activates the *gadd45 α* promoter located in a 153 base pair region between -234 and -81, relative to the transcriptional start site. In addition, this transcriptional induction was abrogated in the presence of H₂O₂ scavengers, suggesting a role for H₂O₂ in the transcriptional control of the *gadd45 α* gene through a Fenton-like free radical mechanism.

Introduction

Inorganic arsenic is a ubiquitous environmental contaminant associated with a multitude of adverse health effects, including hyperkeratosis, hyperpigmentation, diabetes mellitus, cardiovascular disease, reproductive defects, and cancers of several tissues including liver, bladder, skin, lung, kidney, and prostate [1-17]. The most common route of arsenic exposure is the ingestion of contaminated drinking water, which has been documented in many countries at concentrations as high as 3.4 mg/L; more than 3000 times the World Health Organization recommended limits of 10 µg/L [18-23]. In the United States, more than 56 million Americans are estimated to have been exposed to drinking water containing arsenic concentrations that elevate the fatal cancer risk [24].

Although arsenic is poorly mutagenic in rodent and bacterial models, it has been shown to cause DNA adduct formation, DNA-protein crosslinking, and the formation of reactive oxygen species (ROS) *in vitro* [25-27]. In addition, arsenic has been shown to induce an assortment of signaling events including NF-κB activation, AP-1 activation, c-myc overexpression, involucrin repression, and extracellular signal-regulated kinase (ERK) activation, which are involved in such diverse processes as DNA repair, cellular differentiation, cell cycle arrest, and malignant transformation [28-32]. Together, these findings suggest that arsenic may be exerting its carcinogenic effects through an epigenetic mechanism.

Cell cycle dysregulation and/or perturbation is a major mechanism of carcinogenesis. While the signaling pathways involved in the G₁/S phase checkpoint have been well examined, much less is known about the signaling events that occur at the G₂/M phase checkpoint. Previous data from our lab and others have suggested that

arsenic treatment can induce G₂/M phase growth arrest in several different cell lines [33-36]. A major player in G₂/M arrest is the growth arrest and DNA damage-inducible protein 45 α (GADD45 α), which is induced by a variety of stress-induced signals including methyl methanesulfonate (MMS), ultraviolet (UV) irradiation, ionizing radiation (IR), sodium arsenite exposure, and serum starvation [37-42]. In addition, GADD45 α is proposed to play roles in chromatin remodeling and apoptosis [43,44].

Transcription of the *gadd45a* gene is thought to be controlled by both the promoter region and the third intron region and seems to be differentially activated depending on the stimulus [45]. For example, in response to IR, the p53 transcription factor binds to its consensus sequence site in the third intron of the *gadd45a* gene and subsequently interacts with the WT1 transcription factor to enhance gene expression [46]. However, several groups have also suggested that after MMS and UV exposure, the ubiquitous Oct-1 transcription factor binds the promoter region between -101 and -82 base pairs (bp) relative to the transcriptional start site (tss) of the *gadd45a* gene with the help of an unidentified cofactor to activate *gadd45a* transcription in a p53-independent manner [47-50]. Recently, the forkhead transcription factor family member FOXO3a, has also been shown to bind to the forkhead response elements (FHREs) located in the *gadd45a* gene promoter region at -505, -377, and -803 in response to UV, resulting in transcriptional activation [51]. Thus, *gadd45a* gene regulation is complex and may involve many different signaling pathways.

Since GADD45 α is a general stress response protein, we examined the effects of ROS produced in response to As(III) exposure on *gadd45a* gene regulation. We used a human bronchial epithelial airway cell line, BEAS-2B, to examine these effects because

the lung is a target of arsenic induced carcinogenesis, particularly in occupational settings [7]. Our results show that As(III) transcriptionally induces *gadd45a* gene expression from its promoter region via a free radical mechanism involving the formation of H₂O₂.

Materials and Methods

Cell Culture and Media.

The BEAS-2B human lung epithelial cell line was obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's Modified Eagle Medium (Sigma, St. Louis, MO) containing 5% fetal bovine serum (Invitrogen/Gibco BRL, Carlsbad, CA), 1 unit/mL penicillin, 1 µg/mL streptomycin, and 300 µg/mL L-glutamine (Sigma, St. Louis, MO). BEAS-2B cells were passaged when confluency was reached, usually every 4-5 days.

Western Blotting.

Six well tissue culture plates were grown to a density of approximately 1 x 10⁶ cells per well and treated with concentrations of AsCl₃ (Sigma, St. Louis, MO) ranging from 0-50 µM over a time period of 0-24 hours. Phosphate buffered saline (PBS) washed cells were incubated five minutes with 100 µL of MPER mammalian lysis reagent (Pierce Biotechnology, Inc., Rockford, IL) with shaking at room temperature. A portion of each sample's cell lysate (25 µL) was subsequently analyzed for total protein concentration using a BCA Protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL). Pre-cast 4-20% gradient polyacrylamide gels (Invitrogen, Carlsbad, CA) were loaded with 20 µg of total protein for each sample and electrophoresis was performed for approximately 105

minutes at 125 volts using a 1X dilution of 10X Tris-Glycine Running Buffer (Invitrogen, Carlsbad, CA), the XCell *SureLock* Mini-Cell system (Invitrogen, Carlsbad, CA), and a PowerPac 300 power supply (Bio-Rad Laboratories, Hercules, CA). Gels were rinsed with a 1X dilution of 25X Tris-Glycine Transfer Buffer (Invitrogen, Carlsbad, CA) and transferred to a PVDF membrane (Invitrogen, Carlsbad, CA) at 35 volts for two hours using the XCell II Blot Module (Invitrogen, Carlsbad, CA). PVDF membranes were blocked for 1 hour at room temperature with 5% milk buffer in TBS-Tween (Sigma, St. Louis, MO). Membranes were incubated with a 1:200 dilution of GADD45 α (H-165) primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in 5% milk buffer overnight at 4° C on a rocking platform. For β -actin primary antibody (Sigma, St. Louis, MO) and GAPDH primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), dilutions of 1:2000 and 1:200 were used, respectively. Membranes were subjected to three ten minute washes with TBS-Tween (Sigma, St. Louis, MO) and immediately placed in a 1:1000 dilution of an anti-rabbit or anti-mouse IgG AP-linked secondary antibody (Cell Signaling Technology, Beverly, MA) in 5% milk buffer for 2-4 hours at room temperature with shaking. After three ten minute washes with TBS-Tween (Sigma, St. Louis, MO), membranes were immediately incubated with CDP-Star Reagent (New England BioLabs, Inc., Beverly, MA) for five minutes with shaking in the dark at room temperature. Membranes were rinsed with TBS-Tween and exposed to X-ray film (Daigger, Vernon Hills, IL).

Oxygen Consumption.

A Gilson 5/6 Oxygraph (Gilson Medical Electronics, Inc., Middleton, WI) was used to perform oxygen consumption studies. BEAS-2B cells were trypsinized and washed with 1X PBS (Invitrogen/Gibco BRL, Carlsbad, CA) and 4×10^6 cells were re-suspended in a final volume of 1 mL of PBS. 1mM AsCl₃ (Sigma, St. Louis, MO) was added to each aliquot and allowed to incubate for 20 minutes at room temperature. Oxygen consumption rates were calculated using the following equation:

$$\text{nmoles O}_2 \text{ consumed/minute}/10^6 \text{ cells} = \frac{(\text{BP} - 47) \Delta\text{B} (1.835)}{(\text{m})(\text{t})(\text{n})}$$

where BP = atmospheric pressure in mmHg, ΔB = number of vertical blocks traversed on the oxygraph chart paper (rise of experimental slope), m = slope of O₂ standards (409 in this case), t = time in minutes used to determine ΔB (run of experimental slope), and n = n x 10⁶ cells used in the assay. Three replicates of three different samples were averaged together to determine mean O₂ consumption and error bars equal \pm standard error of the mean (SEM).

Electron Spin Resonance (ESR).

1×10^6 BEAS-2B cells were suspended in 500 μL of 1X PBS (Invitrogen/Gibco BRL, Carlsbad, CA) and treated with 1 mM AsCl₃ (Sigma, St. Louis, MO) and/or 2000 U/mL catalase isolated from bovine liver (Sigma, St. Louis, MO). All ESR measurements were conducted using a Bruker EMX spectrometer (Bruker Instruments Inc. Billerica, MA) and a flat cell assembly. 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 $(\Delta H_{pp})^2$, where ΔH_{pp} represents peak-to peak width. The Acquisit program was used for data acquisitions and analyses. Reactants were mixed in test tubes in a final volume of 1.0 mL. The reaction mixture was then transferred to a flat cell for ESR measurement. The concentrations given in the figure legends are final concentrations. Experiments were performed at room temperature and under ambient air. Statistical analysis was performed using Student's t-test and error bars equal \pm SEM.

Construction of GPx1-EGFP and SOD1-EGFP fusion constructs.

pcDNA3.1(+)/GPx1 and pcDNA3/SOD1 constructs were graciously provided to our laboratory by Dr. Larry Oberley's lab at the University of Iowa [52,53]. The GPx1 cDNA was removed from the pcDNA3.1 construct using a *BamHI* (Promega Life Sciences, Madison, WI) and *HindIII* (Promega Life Sciences, Madison, WI) double enzymatic digest. The SOD1 cDNA was removed from the pcDNA3 construct using a *KpnI* (Promega Life Sciences, Madison, WI) and *Apal* (Promega Life Sciences, Madison, WI) double enzymatic digest. Both GPx1 and SOD1 cDNA fragments were isolated from an agarose gel and ligated into a pEGFP-C3 (BD Biosciences – Clontech, Palo Alto, CA) vector in frame with the EGFP protein. Contents of both vectors were verified by DNA sequencing (Biotech Core, Inc., Mountain View, CA).

Transient transfections.

Transient transfections were performed using SuperFect Transfection Reagent (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. Briefly, cells were plated at 50% confluency, and DNA/SuperFect complexes were allowed to enter BEAS-2B cells for three hours. DNA complexes were removed and fresh growth medium was added. Transfection efficiencies of the pEGFP-C3, pEGFP-C3/GPx1, and pEGFP-C3/SOD1 were calculated by counting EGFP (+) cells with a confocal microscope and determined to be roughly 8-10% for all three plasmids.

Quantitative real-time polymerase chain reaction (qRT-PCR).

Total RNA was isolated from approximately 1×10^6 BEAS-2B cells using an RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). Total RNA was quantified using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Quantitect SYBR Green one-step qRT-PCR kits (Qiagen, Inc., Valencia, CA) were used to set up qRT-PCR reactions using 10 ng of total RNA as a template. qRT-PCR experiments were performed on an ABI 7700 qRT-PCR machine (Applied Biosystems, Foster City, CA). Validated GADD45 α primer sets were obtained from Qiagen, Inc. (Valencia, CA) and 18S rRNA primer set controls were obtained from Ambion, Inc. (Austin, TX). Each sample was replicated three times per plate and an average ΔC_t value was calculated. Relative quantitation of GADD45 α mRNA was calculated using the $\Delta\Delta C_t$ method. Error bars represent \pm SEM.

Dual-Luciferase Reporter Assay.

BEAS-2B cells were applied to 12-well tissue culture plates at approximately 50% confluency and transiently transfected with either a full length or deleted GADD45 α promoter-firefly luciferase construct (kind gifts from Dr. Dennis Bruemmer at the University of California – Los Angeles, CA) or a GADD45 α intron 3- firefly luciferase construct (a kind gift from Dr. Albert J. Fornace, Jr. at Harvard University, Cambridge, MA) [54,55]. In addition, each sample was co-transfected with a pRL-TK *Renilla* luciferase vector (Promega Life Sciences, Madison, WI) underneath the constitutive control of the herpes simplex virus thymidine kinase promoter to provide a measure of transfection efficiency for normalization. Constructs were allowed to express for at least 60 hours followed by a 12 hour treatment with 25 μ M AsCl₃ (Sigma, St. Louis, MO). 100 μ L of firefly luciferase substrate was injected into each well of a Lumitrac 200 96-well plate (USA Scientific, Inc., Ocala, FL) containing 20 μ L of cell lysate. Firefly luciferase units were measured by an EG&G Berthold Microplate Luminometer MicroLumat Plus with an automatic injector (EG&G Berthold, Germany). WinGlow software (EG&G Berthold, Germany) was used to obtain readings with a 2.0 second delay and a 10.0 second measurement of luciferase activity and expressed in luciferase units. Luciferase substrates were obtained from the Dual-Luciferase Reporter Assay kit (Promega Life Sciences, Madison, WI). Three replicates were assayed per experiment and the ratio of inducible firefly luciferase activity to basal *Renilla* luciferase activity was averaged for each group. Fold induction was calculated by dividing the average ratios of As(III)-treated samples by the average ratios of samples left untreated. Error bars represent \pm SEM and statistical significance was calculated using Student's t-test. Data shown is representative of three independent experiments.

Results

As(III) exposure induces expression of the GADD45 α protein in a time- and dose-dependent manner. Because GADD45 α expression has been observed in response to many stress conditions including UV irradiation, serum starvation, and alkylating agents, we investigated the effects of As(III) exposure on its induction [32,37,38]. In order to simulate airway exposure to arsenic, an immortalized non-tumorigenic BEAS-2B human lung airway epithelial cell line was used for these experiments. Western blot analysis of GADD45 α in response to increasing concentrations of AsCl₃ revealed a time- and dose-dependent increase in protein abundance (Fig. 1), whereas equal concentrations of NaCl had no effect (data not shown). β -actin abundance remained unaffected throughout the dose and time course of As(III) exposure. Densitometry readings, normalized to β -actin expression, confirmed that GADD45 α protein abundance increased more than 100-fold over untreated BEAS-2B cells 12 hours after 25 μ M As(III) treatment (data not shown). This result demonstrates that GADD45 α protein is induced in BEAS-2B cells in response to As(III) exposure in a dose-dependent manner.

As(III) exposure increases oxygen consumption and \cdot OH production in BEAS-2B cells. Since As(III) has been shown to exert mutagenic effects via reactive oxygen species (ROS) production in several cellular systems, it is possible that the signaling events induced by As(III), specifically GADD45 α induction, are also mediated by a ROS mechanism [56]. In order to address this possibility, BEAS-2B cells were exposed to 1 mM As(III) for 20 minutes and the rate of oxygen consumption was measured by a

Gilson 5/6 Oxygraph. As(III)-exposed BEAS-2B cells showed approximately a three-fold increase in the amount of oxygen consumed *in vitro* (Fig. 2A), suggesting that a free radical mechanism may be occurring in BEAS-2B cells upon As(III) stimulation.

To determine the identity of the radicals produced, we employed electron spin resonance (ESR) spectroscopy. Representative ESR spectra are displayed in Figure 2B. A basal level of $\cdot\text{OH}$, signified by its 1-2-2-1 relative peak height fingerprint, is produced by normal BEAS-2B cells, and was expected as a consequence of normal respiration; however, after a 20 minute exposure to 1mM As(III), the $\cdot\text{OH}$ signal significantly increased (Fig. 2B). Spectral results were quantified by peak height measurements in Figure 2C, where a 2.5- fold increase in $\cdot\text{OH}$ production was observed after As(III) exposure when compared to normal respiring cells. The $\cdot\text{OH}$ signal was attenuated by the addition of 2000 units/mL of catalase, showing that H_2O_2 is a precursor of $\cdot\text{OH}$. These data indicate that $\cdot\text{OH}$ is a major free radical formed in the BEAS-2B cell line in response to As(III) exposure.

GADD45 α protein abundance decreases in response to As(III) in the presence of the H_2O_2 scavenger, GPx1. Because As(III) produced $\cdot\text{OH}$ in the BEAS-2B cell line and GADD45 α is induced in response to many stressors, we hypothesized that its induction may occur through an oxidative stress mechanism. To assess the role of ROS in GADD45 α induction, we constructed glutathione peroxidase 1 (GPx1)-EGFP and superoxide dismutase 1 (SOD1)-EGFP overexpression/fusion vectors and examined their effects on GADD45 α protein abundance. The GPx1 enzyme is a H_2O_2 scavenger, whereas the SOD1 enzyme is a $\text{O}_2^{\cdot-}$ scavenger. GPx1-EGFP, SOD1-EGFP, and empty

vector pEGFP-C3 constructs were transiently transfected into BEAS-2B cells. 48 hours after transfection, cells were treated with 25 μ M As(III) for 12 hours, and subjected to Western blot analysis. Overexpression of the GPx1-EGFP fusion construct reduced GADD45 α protein abundance by at least 50% (Fig. 3) as measured by densitometry and normalized to GAPDH expression (data not shown). Empty vector and SOD1 construct overexpression did not affect GADD45 α expression (Fig. 3), implying that the formation of H₂O₂ is partially responsible for the induction of the GADD45 α protein in response to As(III).

As(III) exposure increases GADD45 α mRNA abundance. To determine how As(III) exposure induces the GADD45 α protein, we examined GADD45 α transcript abundance at several time points using qRT-PCR. A concentration of 25 μ M As(III) was chosen to obtain a maximal effect. Relative quantification of GADD45 α mRNA was performed using the 0 hour time point as a calibration value. As shown in Figure 4, GADD45 α mRNA increases almost 30-fold after three hours of treatment. Although GADD45 α mRNA slightly decreased during the following three time points, it began to increase again at the 24 hour time point, mimicking the GADD45 α protein expression pattern (see Fig.1). Therefore, we reasoned that the *gadd45 α* gene could be transcriptionally induced by As(III) exposure.

As(III) activates transcription of gadd45 α through both the promoter region and the third intron region. To establish whether the increase in mRNA abundance resulted from transcriptional activation or as a consequence of increased mRNA stability, we

examined the effects of As(III) on induction of the *gadd45α* promoter and intron 3 regions. GADD45α promoter-luciferase and intron 3-luciferase transcriptional fusion constructs were transiently transfected into BEAS-2B cells, subjected to As(III) treatment for 12 hours, and assayed for luciferase activity. The GADD45α promoter construct showed a five-fold increase in luciferase activity in response to 25 μM As(III) (Fig. 5). Similarly, the GADD45α intron 3 construct exhibited a four-fold increase in activity (Fig. 5). Neither of the control constructs containing the luciferase gene alone (GVB2 or GL3) showed any discernible difference between the untreated samples and the As(III)-exposed samples, illustrating that both the promoter and intron 3 regions of the *gadd45a* gene are activated by As(III) exposure.

Since H₂O₂ was involved in As(III)-induced GADD45a protein levels, we examined the effects of H₂O₂ on the transcriptional activation of *gadd45a*. GPx1 or a control vector was co-transfected with the aforementioned GADD45α promoter and intron 3 constructs. GPx1 overexpression decreased promoter-luciferase activity by 50% when compared to transfection with the empty vector (Fig. 6). Surprisingly, the control vector itself slightly increased luciferase activity in the *gadd45a* promoter construct. The reason for this phenomenon is unclear. In contrast, the activity of the intron 3 construct after As(III) exposure remained largely unaffected (Fig. 6). These results suggest a role for H₂O₂ in the transcriptional activation of the *gadd45a* promoter region.

As(III) activation of the GADD45α promoter region occurs between -81 and -234 relative to the tss. Given that the GADD45α promoter-luciferase transcriptional fusion construct was activated by the presence of H₂O₂, we wanted to pinpoint which portion of

the promoter region was responsible for the activation. Six GADD45 α promoter-luciferase deletion constructs, named for the length of the promoter region with regards to the transcriptional start site (+1), were transfected into BEAS-2B cells and exposed to 25 μ M As(III) for 12 hours (Fig. 7A). A five- to six- fold increase in luciferase activity was observed in all constructs containing at least 234 base pairs upstream of the +1 site (Fig. 7B). A further deletion of approximately 150 bp (to -81) eliminated most of the luciferase activity. Putative transcription factor binding sites located in this region include consensus sequences for AP-1, MBF-1, Egr-1, Egr-2, Egr-3, and WT1 (-KTS) [57].

We subsequently examined the effects of As(III)-induced H₂O₂ formation on the induction of the promoter deletion constructs. Again, GPx1 overexpression and the removal of H₂O₂ decreased luciferase induction by approximately 50% in all constructs showing more than five-fold induction (Fig. 7C). No change was seen in the smaller constructs. These results suggest that the 153 bp region located between -234 and -81 contains an As(III)-responsive region that is sensitive to the production of H₂O₂.

Discussion

The findings presented here suggest that As(III) exposure induces the transcriptional activation of the *gadd45a* gene through a Fenton-like free radical mechanism (Fig. 8). The Fenton reaction, which is usually associated with an excess of Fe(II), occurs when a transition metal is oxidized during its interaction with H₂O₂, producing an oxidized metal and two molecules of \cdot OH. Our data show that As(III) treatment increases the amount of molecular oxygen consumed by BEAS-2B cells,

indicating that more oxygen is available for conversion to $O_2^{\cdot -}$ and subsequent formation of H_2O_2 . In addition, ESR data shows that the relative amount of $\cdot OH$ increases in response to As(III) exposure, which is alleviated by treatment with the H_2O_2 scavenger enzyme catalase. The removal of H_2O_2 from the intracellular environment by GPx1 overexpression decreases *gadd45a* promoter-luciferase activity and GADD45 α protein expression, further supporting a role for H_2O_2 in GADD45 α induction. Surprisingly, treatment with H_2O_2 alone only weakly induced GADD45 α protein expression when compared to equal concentrations of As(III) treatment, whereas co-treatment with H_2O_2 and As(III) enhanced GADD45 α protein induction (data not shown). Collectively, these results suggest that the formation of $\cdot OH$ through a Fenton-like reaction is at least partially responsible for the induction of *gadd45a* transcription and protein expression in response to As(III) exposure.

The induction of *gadd45a* transcription by the production of H_2O_2 and/or $\cdot OH$ and each molecule's subsequent intracellular activities may partially account for the wide variety of chemical and physical inducers of GADD45 α protein expression. IR and UV irradiation, both potent inducers of GADD45 α expression, also induce $\cdot OH$ formation [37,58]. Additionally, generation of H_2O_2 and $\cdot OH$ via the Fenton reaction can induce the formation of apurinic sites, the same type of DNA lesion induced by another potent GADD45 α inducer, methyl methanesulfonate (MMS) [37,59]. These data suggest that H_2O_2 and/or $\cdot OH$ production may be a key regulator of GADD45 α transcriptional induction.

Because, BEAS-2B cells contain a p53 protein that has been inactivated by expression of the E6 viral protein, *gadd45a* transcriptional activation by As(III) occurs

through a p53-independent mechanism in these cells [60]. Based on the experimental data presented in this manuscript, we have identified several other candidate transcription factors that may be involved in *gadd45a* transcription. One major candidate is the AP-1 transcription factor, since it has previously been identified as an As(III)-activated protein [61,62]. In addition, the yeast homolog of the human AP-1 protein, Yap1, contains a redox regulated domain consisting of disulfide bonds that can mask or expose its nuclear localization sequence, depending on the redox status of the cell [63]. These findings make AP-1 an ideal candidate for ROS-mediated transcriptional activation. Moreover, the 153 bp region of the *gadd45a* promoter-luciferase deletion constructs affected by antioxidant overexpression boasts four possible AP-1 consensus binding sequences [57]. Finally, AP-1 has also been identified as the transcription factor responsible for increasing expression of the DNA damage response gene *gadd153* after As(III) exposure, suggesting that H₂O₂ production and redox status may be a general mechanism for the transcriptional activation of DNA damage response genes [64].

Alternatively, the early growth response 1 (Egr-1) transcription factor may also be involved in H₂O₂-mediated transcriptional activation of *gadd45a*. Egr-1 has recently been shown to induce *gadd45a* transcription in response to UV irradiation [65]. Furthermore, Egr-1 induction occurs three hours after 25 μ M As(III) treatment coincidental with the increase in *gadd45a* mRNA abundance shown in Fig. 4 (D. Bhatia and F. Chen, unpublished data). Testing is underway in our laboratory to determine the effects of Egr-1 on *gadd45a* transcriptional activation in response to As(III) exposure.

In conclusion, we have shown that the *gadd45a* gene is transcriptionally induced by As(III) exposure through the formation of H₂O₂. This induction occurs in the

promoter region of the *gadd45a* gene and the redox responsive element is located in the -234 to -81 base pair region. Future work will focus on the identification of the transcription factor(s) responsible for the oxidative stress-dependent transcriptional induction of *gadd45a*.

List of Abbreviations:

GADD45 α	growth arrest and DNA damage-inducible protein 45 alpha
H ₂ O ₂	hydrogen peroxide
As(III)	arsenite
bp	base pairs
tss	transcriptional start site
mg	milligram
μ g	microgram
L	liter
DNA	deoxyribonucleic acid
ROS	reactive oxygen species
NF- κ B	nuclear factor κ B
AP-1	activator protein 1
ERK	extracellular signal-regulated kinase
G1/S	gap 1/synthesis phase checkpoint
G2/M	gap2/mitosis phase checkpoint
MMS	methylmethane sulfonate
UV	ultraviolet irradiation
IR	ionizing radiation
FHRE	forkhead response elements
mL	milliliter
AsCl ₃	arsenic trichloride
μ M	micromolar

μL	microliter
PBS	phosphate buffered saline
μg	microgram
PVDF	Polyvinylidene fluoride
TBS-Tween	Tris-buffered saline
O_2	oxygen
SEM	standard error of the mean
mM	millimolar
ESR	electron spin resonance
G	Gauss
DPPH	diphenyl-2-picrylhydrazyl
K_3CrO_8	potassium tetraperoxochromate
GPx1	glutathione peroxidase 1
SOD1	superoxide dismutase 1
cDNA	complementary DNA
EGFP	enhanced green fluorescence protein
qRT-PCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
C_t	threshold cycle
$\cdot\text{OH}$	hydroxyl radical
$\text{O}_2^{\cdot-}$	superoxide radical
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

mRNA	messenger ribonucleic acid
MBF-1	metal binding factor 1
Egr-1	early growth response gene 1
Egr-2	early growth response gene 2
Egr-3	early growth response gene 3
Yap1	yeast activator protein 1
gadd153	growth arrest and DNA damage-inducible protein 153

Figure Legends:

Fig. 1. 25 μ M As(III) induces maximal GADD45 α expression at 12 hours.

A dose/time course Western blot of As(III)-exposed BEAS-2B cells show maximal GADD45 α induction after 12 hours of treatment. BEAS-2B cells were exposed to As(III) concentrations of 0, 3.25, 6.25, 12.5, 25, and 50 μ M. 20 μ g of total protein were loaded into each lane and GADD45 α expression was detected with a polyclonal antibody.

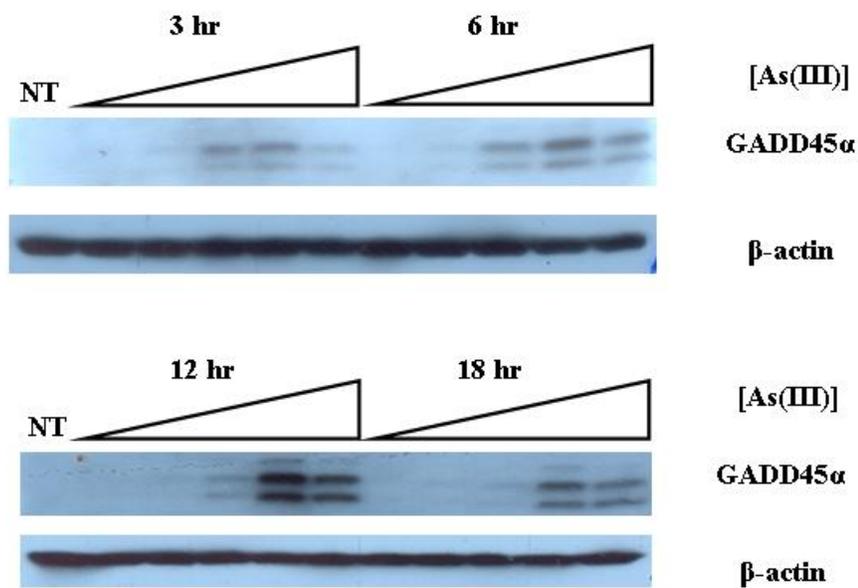


Fig. 2. A. As(III) exposure increases O₂ consumption in BEAS-2B cells.

Aliquots of 4 x 10⁶ BEAS-2B cells were injected into a Gilson 5/6 Oxygraph and the rate of oxygen consumption was measured with or without a 20 minute exposure to 1mM As(III). O₂ consumption in the presence of As(III) was increased approximately three fold.

A.

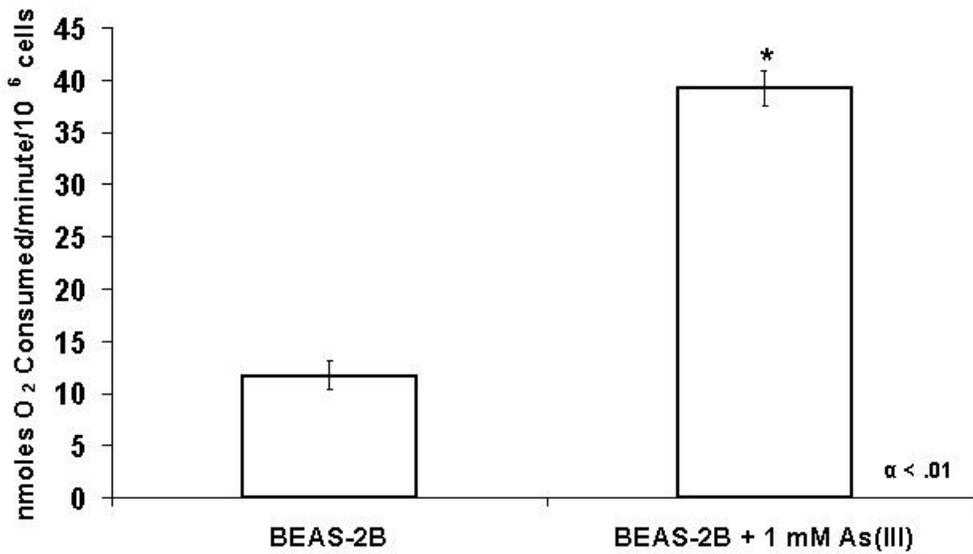


Figure 2B. *As(III) induces the formation of $\cdot\text{OH}$ in BEAS-2B cells.* Formation of $\cdot\text{OH}$ was measured by ESR spectroscopy in aliquots of 1×10^6 BEAS-2B cells with or without exposure to 1mM As(III). Figures shown are examples of spectra of control BEAS-2B cells (left) and As(III)-exposed BEAS-2B cells (right) exhibiting the characteristic $\cdot\text{OH}$ ESR fingerprint.

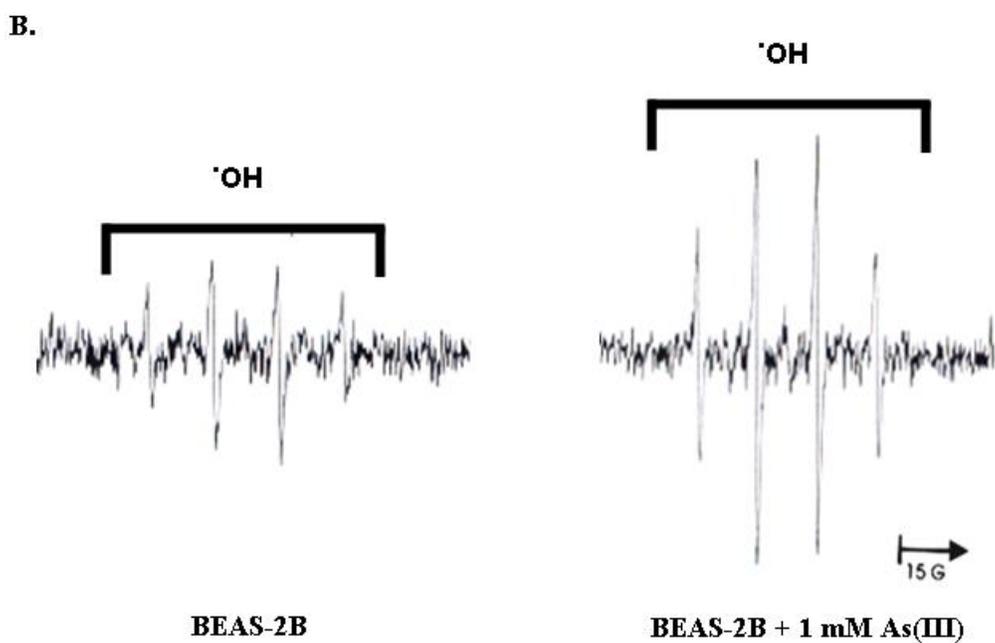


Figure 2C. Graphical representation of ESR peak height. ESR peak heights were measured and quantified as described in Materials and Methods. As(III)-exposed BEAS-2B cells showed a 2.5 fold increase in the amount of $\cdot\text{OH}$ formation which was abrogated by the addition of 2000 units/mL of catalase, confirming the identity of $\cdot\text{OH}$.

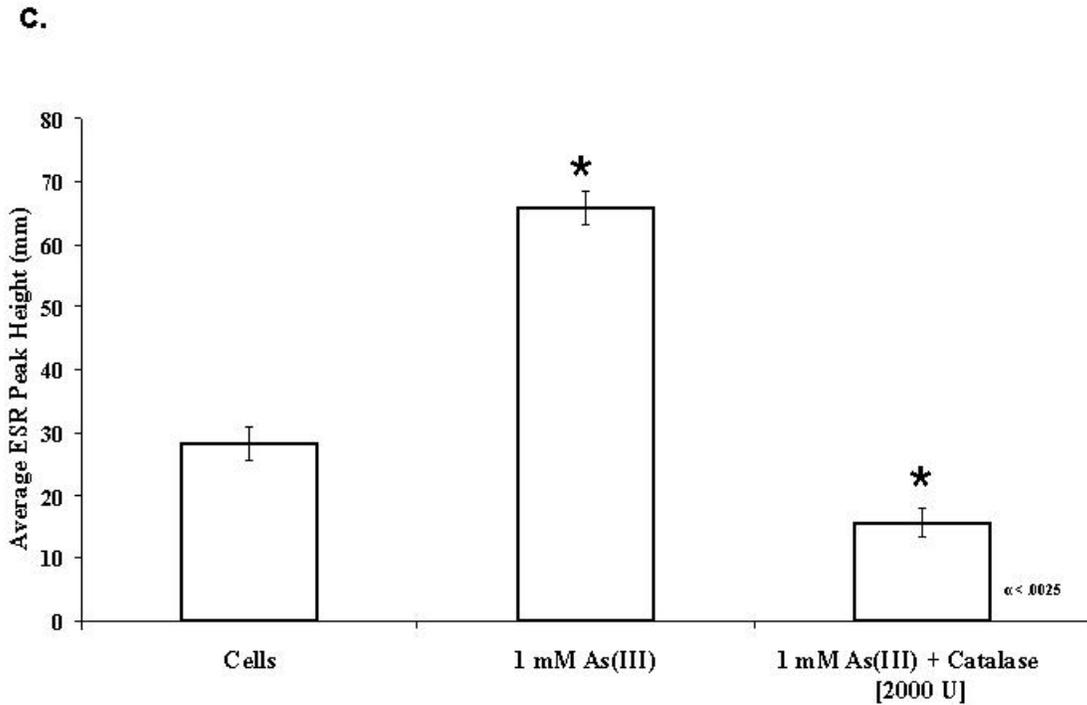


Fig. 3. Overexpression of the H_2O_2 scavenging enzyme GPx1 reduces GADD45 α protein expression. BEAS-2B cells were transiently transfected with a GPx1, SOD1, or C3 empty vector and assayed for changes in GADD45 α expression via western blot. Removal of H_2O_2 by GPx1 overexpression decreased GADD45 α expression by approximately 50%.

25 μM As	-	+	+	+	+
GPx1	-	-	+	-	-
SOD1	-	-	-	+	-
Empty Vector	-	-	-	-	+



GADD45 α



GAPDH

Fig. 4. *As(III)* exposure increases *GADD45α* transcript abundance. Relative qRT-PCR was employed to measure the abundance of the *GADD45α* transcript using the 0 hr time point as a calibrator. Transcript abundance increased approximately 30 fold after a three hour *As(III)* treatment.

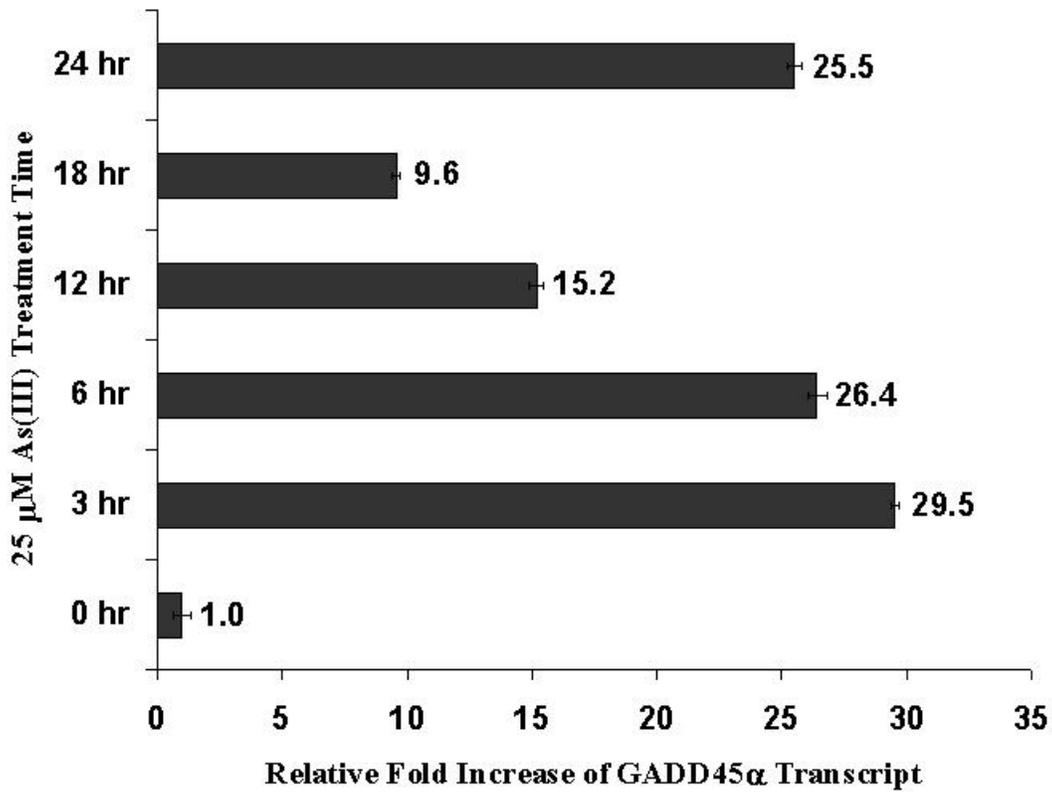


Fig. 5. *As(III)* activates both the *GADD45α* promoter region and the intron 3 region.

A dual luciferase reporter system was used to determine the activation of the *GADD45α* promoter-luciferase (G45) and the intron 3-luciferase (Int3) constructs. Constructs were transiently transfected into BEAS-2B cells and luciferase activity was normalized to an internal transfection control. 5.2 and 4 fold increases in the G45 and Int3 luciferase activity, respectively, were observed in *As(III)*-exposed samples when compared to the untreated control samples.

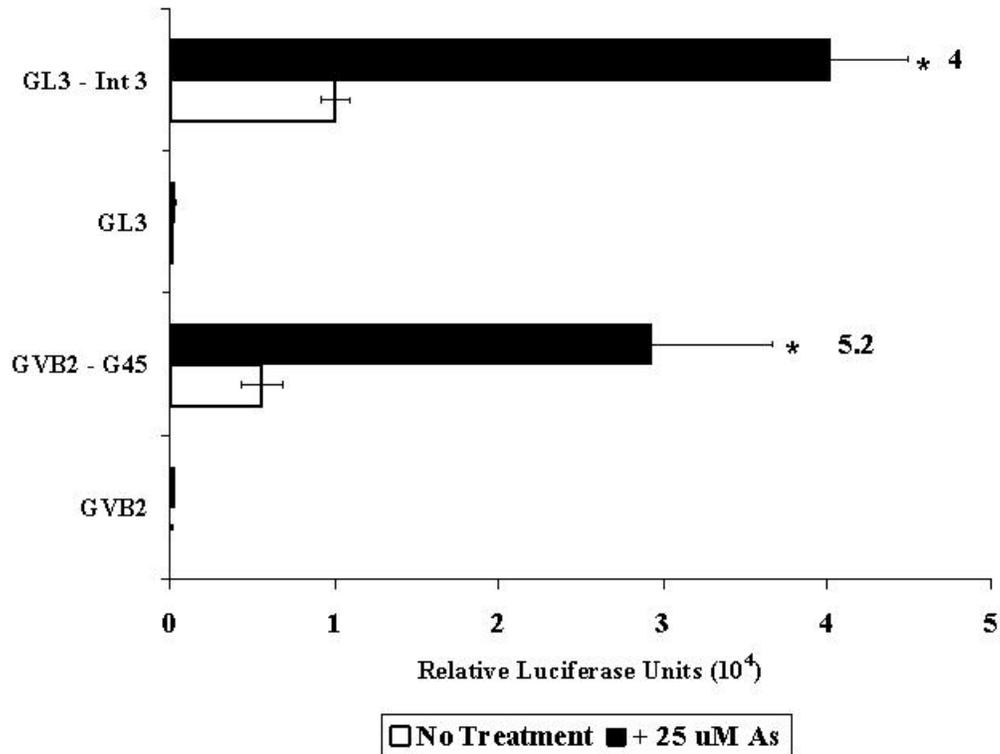


Fig. 6. Overexpression of GPx1 reduces GADD45 α promoter activation, but not intron 3 activation. G45 and Int3 luciferase constructs were transiently co-transfected with either an empty vector or the vector overexpressing the GPx1 protein and exposed to 25 μ M As(III) or left untreated. GPx1 overexpression decreased the G45 promoter luciferase activity by 50%, but seemed to have no effect on Int3 activity.

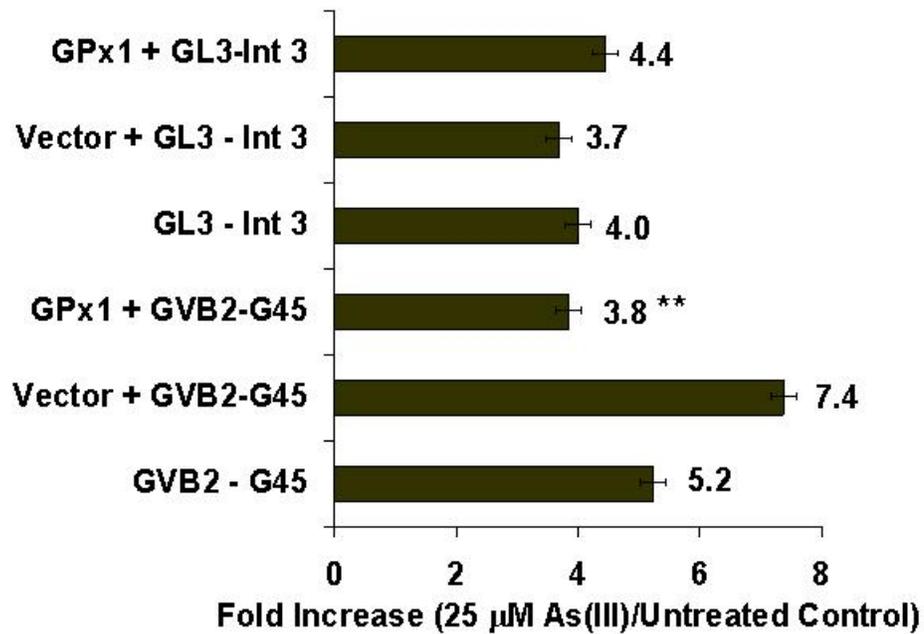


Fig. 7A. *Schematic diagram of GADD45 α promoter-luciferase deletion constructs.*

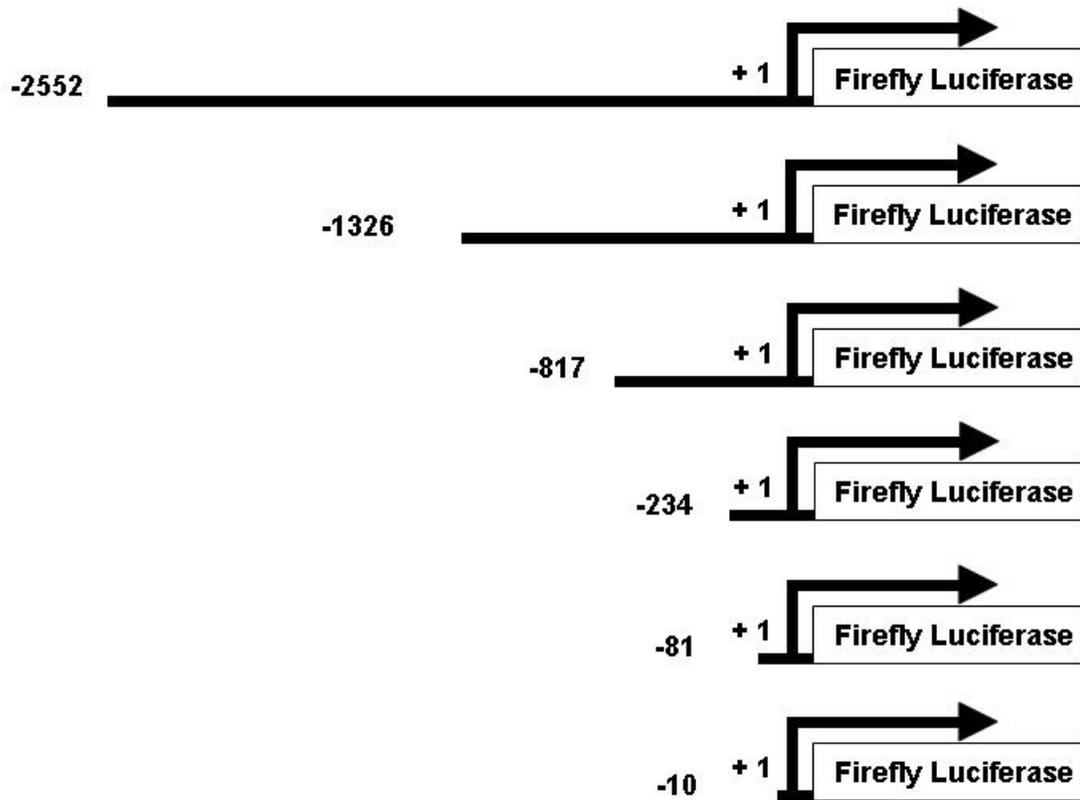


Fig. 7B. *GADD45 α* promoter activation occurs between the -234 and -81 region. The effects of As(III) on the activation of *GADD45 α* promoter luciferase deletion constructs were examined after a 12 hour treatment period with 25 μ M As(III). The full length (-2552), -1326, -817, and -234 constructs all retained an induction of approximately 5-6 fold when exposed to arsenic, whereas the -81 construct showed a loss of activation by As(III).

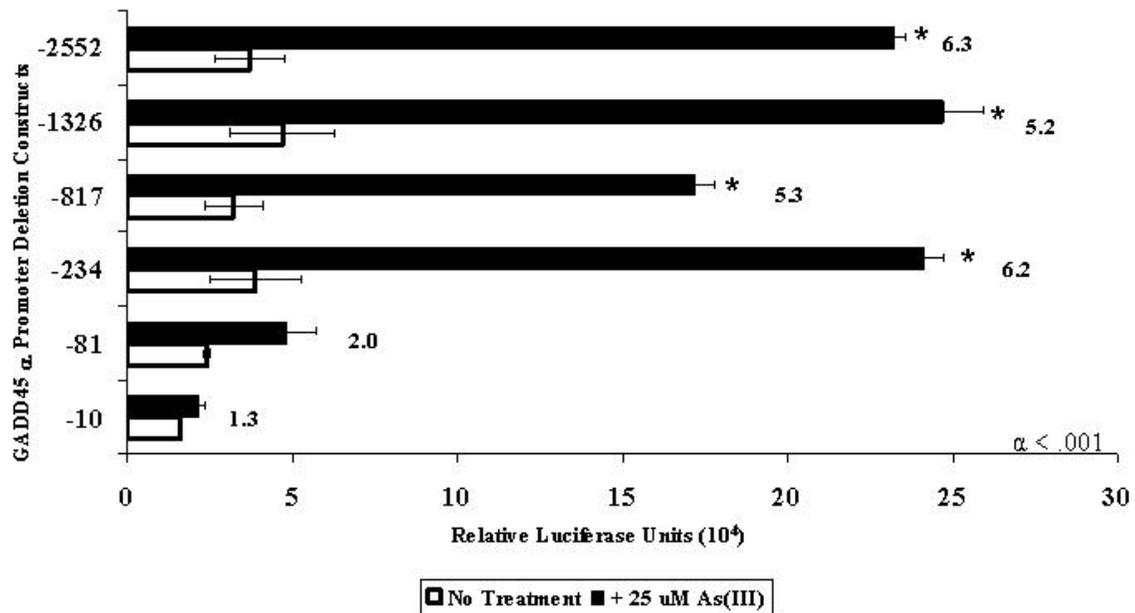


Fig. 7C. *Activation of the full length, -1326, -817, and -234 constructs was reduced by 50% in GPx1-overexpressing cell lysates.*

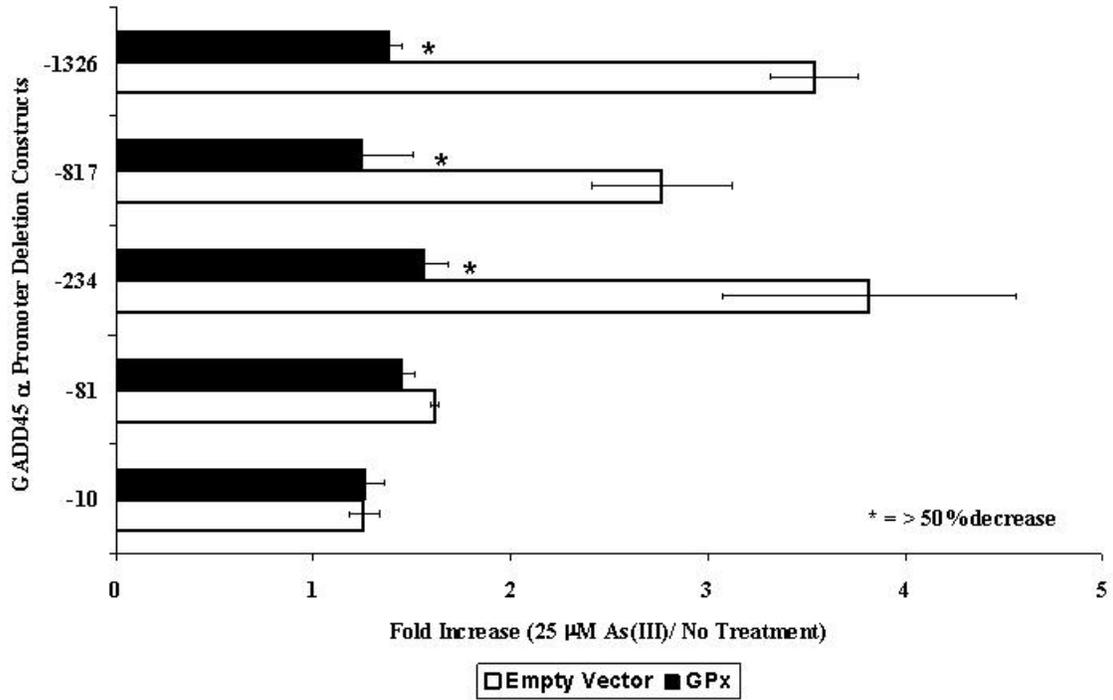
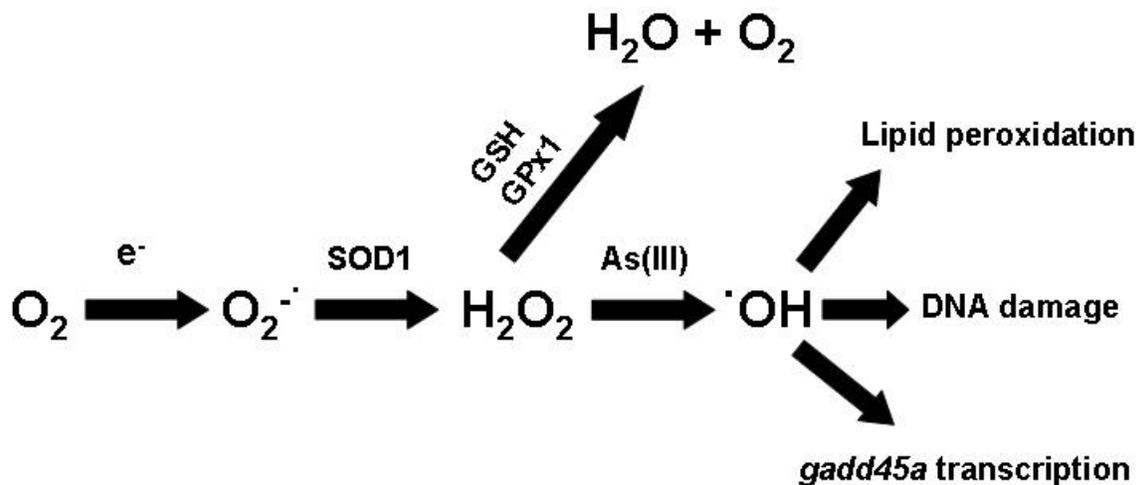


Fig. 8. Proposed ROS pathway involving arsenic. The proposed ROS pathway responsible for the activation of the *gadd45a* promoter region is a Fenton-like reaction in which molecular oxygen accepts an electron, forming $O_2^{\cdot-}$, and is then converted to H_2O_2 by superoxide dismutase. H_2O_2 is then further broken down into water and molecular oxygen, unless the presence of a transition metal, such as As(III), is present. Excess As(III) can then react with H_2O_2 and produce $\cdot OH$, which leads to DNA damage, lipid peroxidation, and the activation of cellular signaling pathways, including the induction of *gadd45a* transcription.



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OVERALL CONCLUSIONS

1. Gene-environment interactions are an integral part of environmental-induced carcinogenesis. The elucidation of the human genome sequence and new techniques that have emerged will play a major role in understanding the molecular mechanisms underlying the carcinogenic action of environmental contaminants.
2. Metal exposure is a major contributor to environmental induced carcinogenesis. Each metal produces its own effects, and these effects are different for each cell type. Identification of each individual mechanism of metal-induced carcinogenesis is necessary in order to obtain effective treatments.
3. Arsenite produces hydroxyl radical in the BEAS-2B cell line.
4. Arsenite induces GADD45 α in a dose- and time-dependent manner.
5. Arsenite can elevate hydrogen peroxide and/or hydroxyl radical production and subsequently GADD45 α protein levels in the BEAS-2B cell line.
6. Arsenite induces hydrogen peroxide-mediated GADD45 α transcription.
7. A 153 base pair region of the GADD45 promoter is regulated by As(III)-induced hydrogen peroxide formation.
8. Arsenite induces GADD45 α expression in a p53-independent manner in the BEAS-2B cell line.
9. The transcriptional induction of the *gadd45a* gene by arsenite seems to occur through a Fenton-like reaction mechanism.

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West Virginia University, Morgantown, WV
Department of Basic Pharmaceutical Sciences
PhD Candidate

University of Notre Dame, Notre Dame, IN
Bachelor of Science, May 2001
Major: Biological Sciences

Honors And Awards

Sigma Xi Graduate Research Competition Winner (2005)
Basic Pharmaceutical Sciences Departmental Teaching Award (2005)
West Virginia University Van Liere Convocation Research Award (2004)
Basic Pharmaceutical Sciences Departmental Graduate Research Award (2003)
Oak Ridge Institute for Science & Education Fellowship (2002)
Centers for Disease Control and Prevention Regular Fellowship (2001 – present)
University of Notre Dame Dean's Honor List (2001)
National Science Foundation Fellowship (2000)
University of Notre Dame Scholar (1997)
Robert C. Byrd Honors Scholarship (1997)

Presentations

Invited Speaker – Chinese Academy of Sciences, Institute for Nutritional Sciences, 2005, Shanghai, China *A Graduate Education at West Virginia University*
Invited Speaker – 3rd International Conference on the Molecular Mechanisms of Metal Toxicity and Carcinogenesis, 2004, Morgantown, WV *As(III)-Induction of the Growth Arrest and DNA Damage-inducible protein 45 α*
Invited Speaker – West Virginia University Van Liere Convocation, 2004, Morgantown, WV *The Role of GADD45 α in G2/M Phase Arrest*
Graduate Research Association of Students in Pharmacy, 2004, Atlanta, GA *The Role of GADD45 α in G2/M Phase Arrest*
Allegheny Erie Society of Toxicology Spring Meeting, 2003, Pittsburgh, PA *GADD45 α is an Oxidative Stress Response Protein*
Experimental Biology, 2003, San Diego, CA *GADD45 α is an Oxidative Stress Response Protein*

Professional Activities

Invited Reviewer for Neurotoxicology and Teratology (2004)
American Association for the Advancement of Science (AAAS) – Member 2004-present
Association for Women in Science – West Virginia University Chapter (AWIS) – Treasurer 2004 - 2005
American Physiological Society (APS) – Member 2003-present
Served as an experimental consultant for researchers at the Chinese Academy of Sciences Institute for Nutritional Sciences, West Virginia University, and the National Institute for Occupational Safety and Health
Student Representative - Graduate Research Advisory Council (2003 – 2004)

**Research
Experience**

CENTERS FOR DISEASE CONTROL AND PREVENTION-NIOSH, Morgantown, WV
PhD Candidate, Xianglin Shi, PhD, September 2001-Present

- Currently conducting independent research involving reactive oxygen species and the effects of arsenic on the induction of the GADD45 α pathway and its role in cell cycle arrest.

THE JACKSON LABORATORY, Bar Harbor, ME
Participant, November 7-13, 2003

- Participated in the “Short Course on Gene Microarray Development and Analysis: Approaches to Heart, Lung, Blood, and Sleep Disorders.”
- Designed, prepared, and analyzed microarray experimental data.

CENTERS FOR DISEASE CONTROL AND PREVENTION-NIOSH, Morgantown, WV

Biological Research Technician, Xianglin Shi, PhD, May 2001 – September 2001

- Conducted independent research regarding the regulatory role of nuclear transcription factor NF- κ B and the G2/M phase cell-cycle checkpoint protein, GADD45 α .

UNIVERSITY OF NOTRE DAME, Notre Dame, IN

Lab Assistant, Jeffrey Schorey, PhD, October 1998 – May 2001

- Conducted undergraduate research in microbiology, immunology, and molecular biology including isolation and purification of a complement activating mycobacterial surface glycolipid.
- Cloned retroviral constructs for subsequent transduction of primary murine bone marrow macrophages.

UNIVERSITY OF NOTRE DAME, Notre Dame, IN

NSF Fellowship, Crislyn D'Souza-Schorey, PhD, Summer 2000

- Conducted undergraduate research in cell biology, including the effects of ARF6 on mycobacterial pathogenesis by retroviral expression of ARF6 in primary murine bone marrow macrophages.

WASHINGTON UNIVERSITY, St. Louis, MO

Lab Assistant, Frederik Lindberg, MD/PhD, Summer 1999

- Conducted undergraduate research in immunology, including production of antigens to make monoclonal antibodies to hSIRP α 1, an NK surface receptor molecule responsible for recognition of CD47 on red blood cells.

**Teaching
Experience**

KAPLAN TEST PREP, INC., Pittsburgh, PA

Teacher, Steven Danyluk, Center Manager, March 2002 – November 2005

- Presented lectures to prepare Kaplan students for standardized testing including the MCAT, GRE, DAT, SAT, and ACT.
- Privately tutored students for the MCAT, GRE, and SAT.

WEST VIRGINIA UNIVERSITY, Morgantown, WV

Teaching Assistant Coordinator, Joseph Ma, PhD, September 2004 – December 2004

Teaching Assistant, Joseph Ma, PhD, September 2003- May 2004

- Organized and managed a group of ten teaching assistants
- Delegated general laboratory duties to ensure that laboratory exercises were executed in an efficient manner.
- Provided laboratory assistance to first year pharmacy students learning basic research techniques.
- Prepared lectures to guide students in the concepts behind laboratory exercises.

UNIVERSITY OF NOTRE DAME, Notre Dame, IN
Teaching Assistant, Michelle Whaley, PhD, January 2001-May 2001

- Guided and assisted second year students conducting independent research projects.

Lab Skills

Biological Research Techniques:

- **Molecular:** western blotting, protein purification, standard cloning, 2DE, quantitative RT-PCR, immunoprecipitation, flow cytometry, ELISA, luciferase reporter gene assays, and site-directed mutagenesis.
- **General & Technical:** Tissue culture, mouse manipulations, HPLC, TLC, oxygen consumption, ESR, and confocal microscopy.

Publications **Bower, J.J.**, Leonard, S.S., Chen, F., and Shi, X. As(III) transcriptionally activates the *gadd45a* gene via the formation of H₂O₂. (submitted)

Bower, J.J., Leonard, S.S., and Shi, X. (2005) Conference overview: Molecular mechanisms of metal toxicity and carcinogenesis. *Mol Cell Biochem* 279:3-15

Bower, J.J. and Shi, X. (2005) Environmental health research in the post-genomic sequencing era: New fields, new challenges, and new opportunities. *J Toxicol Environ Health B Crit Rev* 8:71-94

Leonard, S.S., **Bower, J.J.**, and Shi, X. (2004) Conference overview: Metal-induced toxicity, carcinogenesis, mechanisms and cellular responses. *Mol Cell Biochem* 255:3-10

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Bohlon, S. S., Strasser, J.A., **Bower, J.J.**, and Schorey, J.S. (2001) Role of Complement in Mycobacterium avium Pathogenesis: In Vivo and In Vitro Analyses of the Host Response to Infection in the Absence of Complement Component C3. *Infect Immun* 69:7729-7735.