

GLUTAMATE-CYSTEINE LIGASE MODIFIER SUBUNIT
AS A POSSIBLE MODULATING FACTOR IN METHYLMERCURY-INDUCED
DEVELOPMENTAL TOXICITY

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Abstract

The US EPA recently released a fish-consumption advisory, recommending that pregnant mothers reduce their intake of certain fish. Concentrations of methylmercury (MeHg) in commonly consumed fish have raised concern for the health of women of childbearing age and their developing children. Previous experiments have implicated glutathione (GSH), an endogenous, tri-peptide antioxidant, as an ameliorative factor in MeHg toxicity. GSH biosynthesis is rate-limited by glutamate-cysteine ligase (GCL), a heterodimeric enzyme consisting of a catalytic and a modifier subunit (GCLC and GCLM, respectively). To examine the roles of GCLM and GSH synthesis in MeHg-induced developmental toxicity, experiments were conducted employing *Gclm* knockout and hemizygous mice. Eight breeding pairs were established and on gestational day 14, females were inoculated with MeHg or sesame oil vehicle. The animals were sacrificed on gestational day 17, with dissection of the embryos, placenta, and yolk sacs, and maternal brain, liver, and kidney. The genotype of each embryo was determined and tissues were assayed for *Gclm* transcription and GCLM protein expression. While the scale of this experiment was found to be insufficient for definitive assessment of the roles of GCLM and GSH biosynthesis in MeHg toxicity, the work described here may provide pilot data for the design of future experiments. Additional results from such experiments should provide a better understanding of the fundamental processes involved in MeHg-induced developmental toxicity, and suggest public health strategies for protecting developing children from such injury.

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List of Abbreviations

BSO, buthionine sulfoximine

cDNA, complementary DNA from RNA template

CNS, central nervous system

DNA, deoxyribonucleic acid

EDTA, ethylenediamine tetraacetic acid

GCL, glutamate-cysteine ligase, also γ -glutamyl-cysteine synthetase

GCLC, GCL catalytic (heavy) subunit

Gclc, name of the gene encoding the GCLC protein

GCLM, GCL regulatory (light) subunit

Gclm, name of the gene encoding the GCLM protein

GSH, reduced glutathione

GSSG, glutathione disulfide, oxidized glutathione

HRP, horseradish peroxidase

MeHg, methylmercury

mRNA, messenger RNA

NHANES, National Health and Nutritional Examination Survey

PBS, phosphate-buffered saline

Rfd, reference dose, defined as “an estimate of daily exposure to the human population (including sensitive subpopulations) that is likely to be without a risk of adverse effects when experienced over a lifetime” (Committee on the Toxicological Effects of Methylmercury, 2000).

RNA, ribonucleic acid

RT-PCR, reverse transcription-polymerase chain reaction

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TAE, Tris-acetate-EDTA

TES-SB, Tris-EDTA-sucrose plus serine borate (20 mM Tris, 1 mM EDTA, 250 mM sucrose, 20 mM sodium borate, 2 mM serine, pH 7.4)

Tris, Tris HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride

X-gal, 5-bromo-4-chloro-3-indolyl- β -D-galactoside; substrate for β -galactosidase enzyme

Introduction

Overview

The goal of this research was to derive a greater understanding of the role of glutathione (GSH), and the enzymes of its biosynthesis, in methylmercury-induced developmental toxicity. GSH is an endogenous antioxidant important in the scavenging of free radicals and other reactive oxygen species (ROS) (Meister and Anderson, 1983). Methylmercury (MeHg) toxicity has been associated with increased ROS levels and depleted GSH. GSH synthesis is dependent upon the presence and activity of the required biosynthetic enzymes, as well as the availability of GSH precursors. The rate-limiting enzyme in GSH biosynthesis is the heterodimeric protein, glutamate-cysteine ligase (GCL). GCL transcriptional and enzymatic activities have been observed to increase following exposure to MeHg, driving increased GSH biosynthesis (Griffith and Mulcahy, 1999). Thus, it appears that GCL may play an ameliorative role in MeHg-induced toxicity. It has been hypothesized that functional polymorphisms in the genes encoding GCL may be responsible for GCL of reduced catalytic capacity in some individuals, and subsequently depressed levels of GSH (Willis *et al.*, 2003). As a result of their low GSH status, these individuals are expected to suffer greater toxicity following exposure to pro-oxidant toxicants such as MeHg. In the experiments described here, responses to prenatal MeHg exposures were compared among mice of various genotypes with respect to the modifier subunit of GCL (GCLM).

This work was motivated by the ubiquity of MeHg, particularly in certain commonly consumed fish. The current enthusiasm over the health benefits of fish consumption, reflecting their omega-3 fatty acid content, contrasts with the threat of toxicity posed by MeHg-contaminated fish. The developing fetus represents a human population at high-risk for MeHg toxicity, because of its inherent sensitivity, and because this toxicity is most clearly manifested as developmental abnormalities of the central nervous system (CNS) (*vide infra*, 'Mercury and the Toxicity of Methylmercury'). Endpoints of developmental MeHg exposures range from suboptimal to severe cognitive and motor dysfunction.

Variability in toxicity and the occurrence of fetal injury has been observed among individuals with similar levels of exposure. This may reflect genetic variation, resulting in differential uptake, storage, or metabolism of MeHg among individuals. If found to be a factor in the degree of toxicity experienced, polymorphisms in the genes involved in GSH synthesis could be examined as a possible source of the variability observed in toxic response to MeHg.

The U.S. Environmental Protection Agency (EPA) recently reconsidered annual prenatal MeHg exposure rates, after findings of placental blood MeHg levels as high as three times those of maternal blood (Mahaffey, 2004). Given the recognition that more MeHg may be transmitted to the fetus than previously thought, it can be seen that additional research efforts are merited, in order to improve our understanding of the basic mechanisms underlying MeHg-induced embryotoxicity. Furthermore, the role of gene-environment interactions in this toxicity must be examined more closely. The confluence of how MeHg gives rise to developmental toxicity, and the influence of genetic factors on

those toxic endpoints, can then ultimately be addressed in efforts to protect public health from the threat of MeHg toxicity.

Background

Nutritional Health During Pregnancy

In February of 2004 the U.S. EPA reassessed estimates of prenatal MeHg exposure rates, suggesting that 1 in every 7 births may be affected, according to Mahaffey, by exposures above the reference dose (Rfd) (Mahaffey, 2004). This translates to 630,000 individuals affected by prenatal MeHg exposures annually (Mahaffey, 2004). This reassessment was prompted by NHANES data that found an average ratio of 1.7:1 for umbilical to maternal blood mercury levels (Mahaffey, 2004). Prior to these findings, placental and fetal blood mercury concentrations were believed to be lower than those of the mother. Given these new data, the EPA has been forced to reevaluate their estimates of prenatal exposure rates, as well as the endpoints of those exposures. This finding has additionally driven the EPA, in conjunction with the FDA, to release an up-to-date fish consumption advisory for pregnant and nursing women, women who could become pregnant, and young children. This new advisory recommends limiting low mercury-content fish and shellfish intake to a total of 12 ounces weekly, and entirely avoiding high mercury-content fish, such as shark, swordfish, king mackerel, and tile fish (EPA, 2004).

The developing embryo is particularly sensitive to chemical assaults. As MeHg is passed from mother to child, the embryo experiences a higher ratio of MeHg concentration to body mass than would an adult. Additionally, MeHg exposures can significantly interfere with neuronal development and migration in the developing embryo (*vide infra*, 'Mercury and the Toxicity of Methylmercury') (EPA, 1997). When epidemics of MeHg poisoning occurred in Japan and Iraq, infants with severely disturbed CNS development were often born to women who showed no signs of toxicity during pregnancy, illustrating effective placental transport of MeHg (Amin-Zaki *et al.*, 1974; Harada, 1977).

Each mother hopes to reduce the risks of such toxicity, and attempts to make the "right" choices concerning her pregnancy, in order to protect the health of her child. One primary decision faced by prospective mothers is that of the diet that will be observed during the course of the pregnancy. As many birth defects have been tied to maternal nutritional deficiencies, obstetricians attempt to instill in their patients a sense of the importance of healthy eating practices. The consumption of fresh fruits and vegetables is recognized as important for the health of both the adult and the developing human, in terms of its delivery of vitamins, antioxidants, and fiber. Fruits and vegetables may, however, also contain pesticide residues damaging to the developing fetus. Thus, some dietary sources of nutrients may contain contaminants that pose threats to the development of the child. Omega-3 fats are a pertinent example of developmentally important nutrients derived from dietary sources sometimes contaminated with a known fetotoxin -- MeHg. Clearly, dietary choices compel prospective mothers to conduct cost-benefit analyses with

respect to the health of the developing child. If the public health community were able to supply prospective mothers with more information about determining risk, these cost-benefit analyses might prove more effective, and yield results including reduced birth defect rates, and consequent reduced annual economic costs for medical care of those birth defects.

Nutritional Benefits and Costs of Fish Consumption

In recent years, nutritionists have encouraged the American public to decrease saturated fat intake and increase intake of monounsaturated and polyunsaturated fats, by replacing dietary red meat with fish. The omega-3 fatty acids comprise one class of such biologically important, “good” fats, and are present in appreciable quantities in the tissues of certain fish, in nuts, and in the grain, flax. These essential fatty acids cannot be synthesized in the body, but must be obtained from the diet. Adequate dietary intake of these molecules is essential for the structure and function of many human tissues. Cells of the CNS, for example, require omega-3 fatty acids as significant structural and functional membrane elements (Hornstra, et al., 1995).

Optimal consumption of omega-3 fatty acids is especially important during pregnancy for proper development of the CNS. The utilization of these compounds in fetal development is reflected in the falling levels of omega-3 fatty acids in mothers over the course of pregnancy (Hornstra, et al., 1995). Additionally, correlations between omega-3 fatty acid-deficient diets of mothers and developmental disorders in their

offspring have been observed. The omega-3 fatty acid believed to be of the greatest nutritional significance is docosahexaenoic acid (DHA), which is required for proper development of the brain and CNS (Horrocks and Yeo, 1999). DHA deficiency in pregnant mothers is associated with such conditions as attention deficit hyperactivity disorder, cystic fibrosis, and phenylketonuria in their offspring (Horrocks and Yeo, 1999). In rats, DHA deficiencies have been linked to impaired learning and memory (Greiner *et al.*, 1999). Another interesting note about DHA is its apparent effects on monoamine neurotransmission and behavior in rats (Chalon *et al.*, 1998). This finding suggests that MeHg-toxicity studies performed on populations having high dietary fish intake, might be confounded by improved scoring on tests of cognition as a result of high omega-3 fatty acid intake.

Many adults attempt to increase their intake of these important components of the diet by stepping up their fish consumption, because fish are such a rich source of these essential fatty acids. However, fish, while low in saturated fats and often high in omega-3 fatty acids, are known to be capable of incorporating certain toxicants into their tissues over the course of their lives (Laws, 2000).

Fish participate in extensive and complicated food webs (Figure 1). Large fish caught and consumed by humans are usually the top predators of marine food chains. Certain compounds, which may neither be metabolized nor excreted by the predator, are often encountered in the diet of these fish. Such compounds cannot exit the body of the animal. Additionally, these pollutants can enter fish through the passage of contaminated water over the gills. For these reasons, the largest and oldest fish, high on aquatic food chains, have had the greatest exposures to toxicants and usually exhibit the highest tissue

concentrations of such pollutants (Laws, 2000). This process of toxicant concentration is termed bioaccumulation, or biomagnification, and is not observed in terrestrial food webs to the extent seen in aquatic food webs, because of fewer terrestrial trophic levels. Fish commonly exhibiting high MeHg tissue levels include large predatory species, such as tuna, swordfish, and shark, among others (EPA, 2004).

QuickTime™ and a TIFF (Uncompressed) decompressor are needed to see this picture.

Figure 1. Methylation and Bioaccumulation of Mercury. The diagram shows cycling of mercury and mercury-containing compounds in aquatic systems, including deposition,

methylation, biomagnification up aquatic trophic levels. Large predatory fish, with high tissue concentrations of mercury, are consumed by terrestrial predators, here. Adapted from USGS.

Mercury and the Toxicity of Methylmercury.

Mercury is a heavy metal, present naturally in the Earth's crust and is found in both marine and fresh water systems, as well as in the atmosphere. Mercury is anthropogenically introduced to the environment primarily through the burning of coal, certain mining practices, and the disposal of medical waste, among other practices. Evidence suggests that mercury levels in the atmosphere and surface ocean may have tripled over the last century due to anthropogenic emissions (Mason, *et al.*, 1994). Between 1990 and 1996, however, total gaseous mercury appeared to decrease by about 17%, and has since remained constant (Slemr *et al.*, 2003). While these data do suggest a significant improvement in atmospheric quality with respect to mercury, Slemr *et alii* posit that they demonstrate that the extent of anthropogenic mercury input to the environment has been underestimated (2003).

Mercury generated by anthropogenic sources may enter waterways via effluent or run-off, or enter the atmosphere through volatilization. Atmospheric mercury eventually "rains out" and may be deposited in aquatic systems. In these bodies of water, elemental mercury (Hg^0) is organified by aquatic microorganisms, through a biologically-mediated alkylation process (Figure 1). Although elemental and inorganic forms of mercury are toxic to humans, organic mercury-containing compounds pose the greater public health threat. Methylated mercury, or MeHg, is the primary form of organified mercury which bioaccumulates up aquatic trophic levels, to concentrate in the tissues of large predatory fish (Bache *et al.*, 1971). This compound can considerably damage human health

following high-dose exposures, or lower-dose chronic exposures. The primary means of human exposure to MeHg is through the consumption of contaminated fish. Other means of exposure include the ingestion of contaminated foodstuffs or water, in addition to extended exposure to mercury-containing dental amalgams and certain medical procedures. MeHg exhibits both a lipophilicity and a tendency to target the nervous system, that together enable ready passage across blood-brain and placental barriers (Ariza *et al.*, 1999). As a result, diseases arising from MeHg exposures include both behavioral and functional neurological disorders in adults and serious developmental disorders of the CNS in those exposed during gestation or early childhood. Congenital Minamata disease -- the result of prenatal exposures to MeHg-contaminated fish from Minamata Bay -- was marked by delayed behavioral and psychological development, cognitive deficits, disturbances in gait, speech, hearing, visual field, and even an inability to support the head (Harada, 1977).

The means by which MeHg gives rise to toxicity are still not well understood. An association between MeHg toxicity and oxidative stress has been observed in numerous studies. In the brain, MeHg is demethylated to inorganic mercury, a process that may give rise to free radicals (Committee on the Toxicological Effects of Methylmercury, m, 2000). Additionally, heavy metals such as mercury appear to increase rates of free-radical-producing reactions and lipid peroxidation. Lund found that rat kidney mitochondria exhibited depolarized inner membranes when exposed to mercuric compounds (HgCl_2), leading to increased rates of mitochondrial hydrogen peroxide production (1993). These cellular alterations, however, may be mediated by the depletion of free antioxidants -- by complexation of MeHg with GSH sulfhydryl groups,

rendering GSH unavailable -- rather than by direct action of the heavy metal (Rabenstein, 1989; Halliwell and Gutteridge, 1989). These findings suggest that the oxidative stress observed in MeHg toxicity arises both from increases in cellular hydroperoxide levels, and from decreases in intracellular levels of the antioxidant GSH. The inhibition of certain enzymes may also be involved in MeHg toxicity, as alkyl mercury compounds have a high affinity for the sulfhydryl groups (-SH) found in many enzyme active sites (Ariza *et al.*, 1999). While the exact mechanism by which MeHg induces toxicity is not clearly understood, it appears to involve a combination of the effects discussed, culminating in oxidative stress.

Whatever the mechanism leading to oxidative stress, the injury to cells in MeHg toxicity is associated with increased production of reactive oxygen species (ROS) in the cell. These compounds include such species as hydrogen peroxide, superoxide anion, singlet oxygen, and hydroxyl radical, among others. Heightened levels of ROS produce a state of oxidative stress within the cell. Chronic or recurrent oxidative stress has been linked to neurodegenerative diseases, cardiovascular disease, arthritis, cancer, and normal aging (Ariza *et al.*, 1999). Such oxidative stress is toxic to the cell and can give rise to mutations in DNA, inappropriate induction of apoptosis, and other deleterious effects. ROS produced in the vicinity of DNA may alter nucleotide structure and appropriate base pairing, and lead to lesions of the DNA, which may ultimately prove lethal to the cell (Wallace, 1997). Many of these effects severely retard proper fetal development, and the carefully orchestrated sequence of growth, migration, differentiation, and apoptotic events. DNA damage by oxidation, therefore, is likely to be most detrimental to cells that are actively dividing and organisms that are actively developing.

Modest concentrations of ROS are present in the cell under normal conditions, generated within mitochondria during normal metabolism and oxidative phosphorylation. When these compounds are found in excess or in subcellular locations where they may inflict damage upon the cell, however, intracellular antioxidant mechanisms are utilized in order to reduce injury to cellular components.

Possible Roles of Antioxidant Glutathione in Minimizing Toxicity

Antioxidants are molecules which possess the capacity to diminish the impact of oxidative stress in tissues, by deactivating reactive oxygen species. These compounds can be exogenous -- produced outside of the body and delivered to the body via dietary means -- or endogenous -- produced within the body. An endogenous antioxidant of particular interest with respect to metal toxicity is GSH.

GSH is a tripeptide thiol that exhibits antioxidant properties. It is the predominant endogenous antioxidant in the cell and is of significant importance in protecting the cell from harmful effects of oxidants. GSH is also referred to as gamma-glutamylcysteinylglycine, a name deriving from the fact that the glutamate residue is bound to cysteine at its gamma carbon. Composed of the amino acids glutamate, cysteine, and glycine, this low molecular weight compound may also act in cellular cysteine storage, as well as in the transport of other amino acids (Figure 2). Additional intracellular roles of GSH include the maintenance of ascorbate in its functional state, the conjugation of xenobiotics, and the preservation of the catalytically active states of thiol-

enzymes (Griffith, 1999). When hydroperoxides are reduced to water, GSH is oxidized to form oxidized glutathione (GSSG), a glutathione dimer in which the two tripeptides are bound at the sulfur atoms of each cysteine residue. The formation of this disulfide bond occurs in the glutathione peroxidase-catalyzed reduction of hydroperoxides, in which GSH is a cofactor (Figure 2). GSSG is returned to the pool of reduced glutathione, GSH, by glutathione reductases. The antioxidant properties of GSH, in addition to the ability to complex mercury at its sulfhydryl group, support its conjectured role in MeHg detoxification and account for its interest to MeHg researchers.

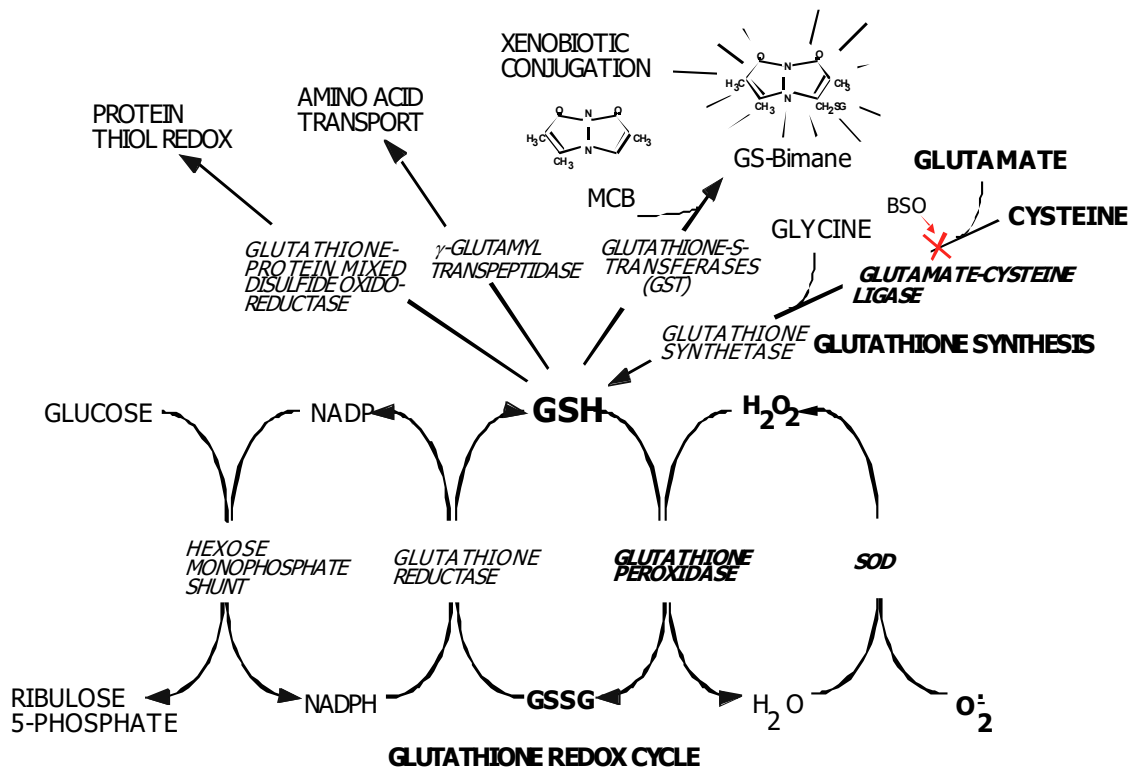


Figure 2. Glutathione Metabolism. The schematic shows a simplified overview of GSH metabolism and intracellular functions. Diagram elements pertaining to this text are seen in bold face. Adapted from T.J. Kavanagh.

Expression of endogenous antioxidants, such as GSH, is influenced by both environmental and genetic factors. While GSH is neither a protein, nor coded for by particular genes, the proteins which catalyze its biosynthesis are gene products. Thus, GSH availability is determined in part by genetic factors.

Glutathione Biosynthesis and Glutamate-Cysteine Ligase

The biosynthesis of GSH occurs in a two-step fashion. The first step is rate-limiting and is catalyzed by the enzyme GCL (Figure 3). Because it is the limiting factor, GCL activity is the primary determinant of total GSH synthesis. GCL is a heterodimeric enzyme consisting of catalytic and modifying subunits encoded by independent genes on separate chromosomes. The catalytic subunit (GCLC) is a 72 kDa polypeptide which catalyzes the ligation of the peptides glutamate and cysteine. When associated with GCLC, the 27 kDa modifying subunit (GCLM) improves the catalytic efficiency of GCLC (Griffith, 1999). The kinetic properties of enzymes such as GCL are usually described in terms of K_m and K_i . The Michaelis constant, K_m , for a particular enzyme is the substrate concentration at which the velocity of enzyme activity is one half of the maximum enzyme velocity. Enzymatic velocity is given as the number of moles of product formed per second per mole of enzyme active site. GCLM improves catalytic efficiency of GCLC by lowering its K_m for glutamate, thereby facilitating greater enzymatic velocity at lower substrate concentrations. K_i is the enzyme inhibition constant, or dissociation constant for inhibitor binding. In this case it is a measure of the

inhibition of the enzyme (GCL) by the end product of the biochemical pathway in which it participates. Because GCL is feedback inhibited by GSH, GCLM improves catalytic efficiency by increasing the K_i of GCLC for GSH, effectively reducing the sensitivity of GCL to the final end product, and requiring greater concentrations of GSH in order to induce feedback inhibition.

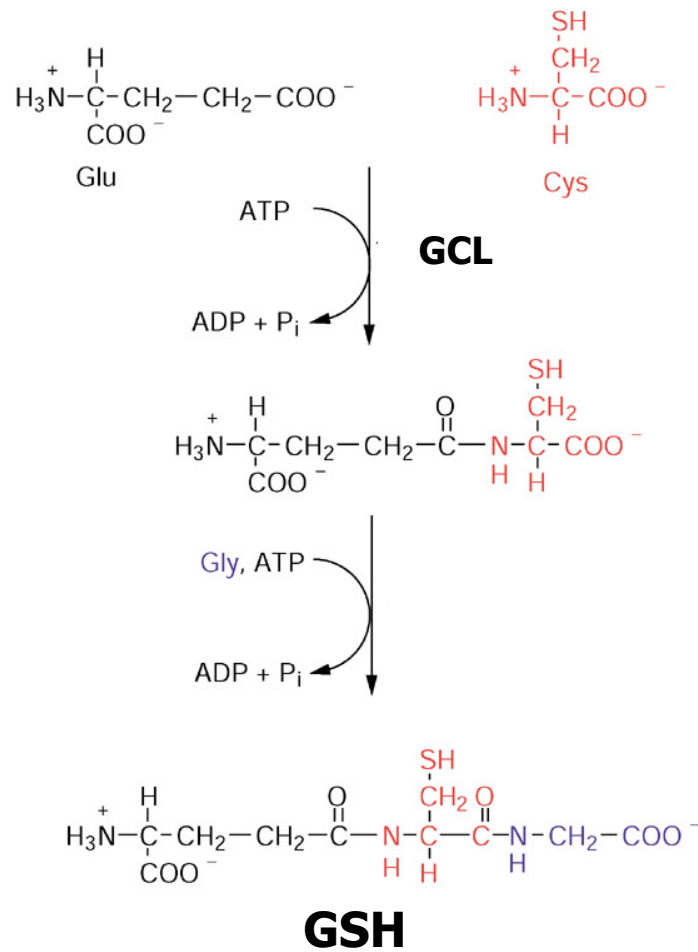


Figure 3. Glutathione Biosynthetic Pathway. The above schematic shows the two-step biosynthesis of GSH. The first step is denoted by the upper arrow, showing the ligation of glutamate to cysteine, as catalyzed by GCL. The end product of this synthetic pathway, GSH, can be seen at the bottom of the diagram.

GCL activity has been observed to increase under conditions of oxidative stress, as have transcription of *Gclc* and *Gclm* genes (Woods *et al.*, 1995). Pro-oxidants and heavy metals lead to the depletion of GSH. This may occur through the production of excess GSSG and its excretion from the cell. Alternatively, as can occur with MeHg, complexes may be formed through interaction with GSH sulfhydryl groups and these complexes may subsequently be excreted. It is likely that the depletion of GSH by these agents leads to upregulation of *Gclc* and *Gclm* genes, in response to the consequent increase in oxidative stress. Because the *Gclc* (-/-) knockout is embryonic lethal and animals do not survive to parturition, a functional animal model for significant GSH deficiency cannot be provided through the knockout of the *Gclc* gene (Dalton, 2000). Yang *et alii* successfully produced viable *Gclm* (-/-) mice which exhibit significantly depressed levels of GSH, as well as increased sensitivity to oxidants such as H₂O₂ (2002). These mice represent a model for GSH deficiencies and compromised ability to respond to oxidative stress, as well as a means to understanding the role of GCLM in GSH synthesis, and in the states of oxidative stress associated with MeHg toxicity (Yang *et al.*, 2002).

Exposure to agents inducing oxidative stress, such as MeHg, appears to up-regulate the *Gclm* and *Gclc* genes; mRNAs for *Gclm* and *Gclc* have been seen to increase 10-fold and 2-fold, respectively (Thompson *et al.*, 2000; Solis *et al.*, 2002). This suggests a correlation between the presence of such oxidative agents in the cell and increased rates of transcription of these genes. It is thought that the promoter region of the *Gclm* gene, which appears to contain an inverted electrophile response element (an ERPE, a DNA motif involved in transcriptional activation by oxidants), may be

responsible for the recognition of the oxidant and the up-regulation of the gene, but initial experiments have not yet substantiated this suggestion (Solis *et al.*, 2002).

It is clear that in the presence of MeHg, GSH levels are diminished, likely as a result of both oxidation and the formation of complexes with mercury. Transcription of *Gclm* and *Gclc* then increases, leading to increased levels of GCL, and thus increased capacity for GSH synthesis (Diaz *et al.*, 2001). When GSH complexes with MeHg (demonstrated in the kidney) it is unavailable to maintain redox balance and consume ROS (Diaz *et al.*, 2001). Such oxidants appear to leak out of mitochondria and, in the absence of combative antioxidants, spread throughout the cell and cause a state of oxidative stress (Meister, 1995). This likely leads to much of the toxicity associated with MeHg exposure. Meister (1995) induced GSH deficiency in animals by administration of buthionine sulfoximine (BSO), which inhibits GCL by irreversibly binding to its active site (Figure 2). The animals exhibited severe and widespread mitochondrial damage, due to the accumulation of ROS produced within mitochondria (Meister, 1995).

Because the GCLC and GCLM proteins required for synthesis of GSH are genetically determined, variability can be expected, both in the functionality and expression of GCLC and GCLM, as well as in the levels of GSH synthesized among individuals. Variability can be due to both genotypic factors (multiple, even defective, GCL alleles, have been recognized), and non-genotypic factors, as GCL expression is known to vary widely among adult humans, including such at risk populations as pregnant mothers (Pamphlett *et al.*, 1998; Dalton *et al.*, 2000). One study, in eastern Australia, sought to determine the role of metal toxicants in the development of sporadic Motor Neuron Disease (MND) by examining genetic variability influencing GSH and

metallothionein metabolism among MND patients and control cases (Pamphlett *et al.*, 1998). The genetic variability affecting GSH synthesis is clearly being examined by the scientific community as a possible factor in a broad spectrum of human health conditions, including those suspected to involve environmental risk factors, in addition to gene-environment interactions.

Study Relevance

Minamata Bay, which provided the dietary staples of fish and shellfish to Minamata, Japan, was contaminated with MeHg from the 1940s until 1976. Cases of MeHg poisoning, or “Minamata Disease,” were documented between 1953 and 1973, but it was not until 1976 that the Japanese government responded by removing the mercury-enriched sediments of Minamata Bay and forcing private industry to discontinue the dumping of mercury-containing effluent (Irukayama, 1977). The residents’ exposure to MeHg, while horrifying and unfortunate, provided a case study for the health effects and disease endpoints of such exposures. While most adults and children in Minamata were exposed and many did become ill, those exposed *in utero* were among the most severely affected. Mothers sometimes exhibited no signs of toxicity themselves, but gave birth to grossly malformed, or neurologically or cognitively compromised infants. These observations, in addition to laboratory animal experiments, suggested that MeHg accumulated by the mother may be passed to the developing embryo, effectively reducing the mother’s burden of the toxicant, at the cost of injury to the embryo (Ariza *et al.*,

1999). This toxicant is able to cross the placental barrier and concentrate in fetal tissue due to its lipophilicity (Komulainan, 1988). For this reason, MeHg exposures pose a particularly serious threat for pregnant mothers and their developing children.

In the 1970s yet another epidemic of MeHg poisoning occurred in Iraq, when grains treated with fungicides containing MeHg were ground into flour and consumed. Children exposed *in utero* during this epidemic exhibited similar developmental disorders to those observed in Japan, including impaired cognitive and motor functioning, such as delayed walking and talking, and cerebral palsy (Committee on the Toxicological Effects of Methylmercury, 2000). Increased rates of spontaneous abortion, as well as decreased brain size and altered brain architecture in the infants, were observed in both Japanese and Iraqi cases (Ariza *et al.*, 1999). All of these disturbances in development have had lifelong consequences for the victims, involving both impaired health and reduced cognitive functioning.

Given this knowledge and the current rise in fish intake, the risk of MeHg-induced toxicity to developing embryos merits greater attention by the scientific and medical communities. As GSH appears to be of primary importance both in the detoxification of MeHg and the development of MeHg toxicity, GCL and GSH synthesis seem appropriate topics for study in addressing the mechanisms of this toxicity. The variation observed in toxic response to MeHg suggests that genetic variability may be a key determinant of risk to individuals. Indeed, studies have already shown some allelic diversity in the genes for the subunits of GCL (Dalton *et al.*, 2000; Willis *et al.*, 2003). As mentioned previously, studies have already been undertaken to examine population

polymorphisms for *Gclc* and *Gclm* genes, and their relationships with diseases such as MND (Pamphlett *et al.*, 1998).

To address the roles of GCLM and GSH synthesis in MeHg-induced embryotoxicity, an experiment was designed to examine the effects of prenatal MeHg exposure among mice of various *Gclm* genotypes. In these studies both the extent of toxicity and the responsiveness of the *Gclm* gene to MeHg, and associated oxidative stress, could be examined.

Methods

Animals

A stable population of *Gclm* null (-/-) mice has been established at the University of Washington, in Seattle, Washington. To produce these knockout animals, the *Gclm* gene was first disrupted in mouse embryonic stem (ES) cells. Through gene-targeted homologous recombination, the first of the seven exons was replaced with an in-frame fusion protein, β -galactosidase/neomycin phosphotranferase, also called β -Geo (Figure 3). The neomycin phosphotranferase domain of the fusion protein allowed positive

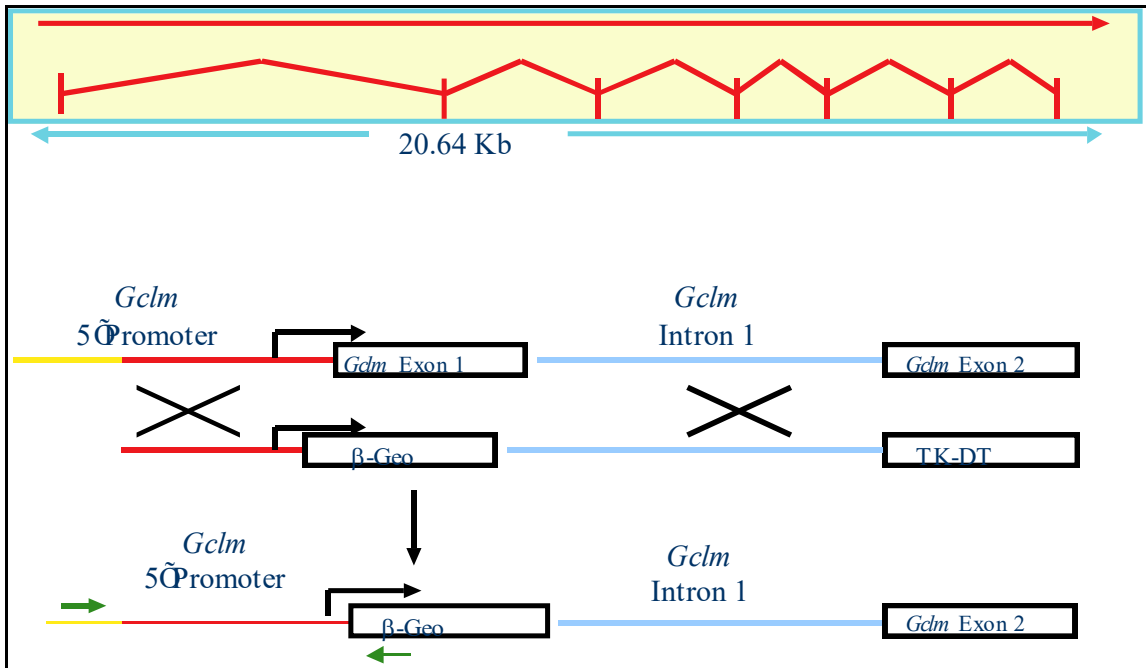


Figure 4. *Gclm* Knockout Mouse Construct. The *Gclm* gene is 20.64 Kb in length and contains seven exons, as shown in the upper portion of the figure. In the disruption of the gene, the first exon was replaced with the fusion protein β -galactosidase/neomycin phosphotranferase (β -Geo) through targeted homologous recombination. Adapted from T.J. Kavanagh.

selection for the first cross-over event; cells which had undergone this first cross-over event would grow in a medium containing G-418. To insure the desired two cross-over events occurred, a thymidine kinase-diphtheria toxin (TK-DT) sequence was included in the construct, downstream of the region of homology. If only the first cross-over event occurred, TK-DT would be expressed. This would act as double negative selection, as cells would be killed by application of the drug ganciclovir and/or diphtheria toxin expression. (Ganciclovir is a chain-terminating nucleotide analog, which, in the presence of this thymidine kinase, can ultimately be converted to the triphosphate form and lead to loss of cell proliferation, due to interference with proper DNA replication.) If both cross-over events occurred, ES cells would survive in media containing both ganciclovir and G-418. These successfully transformed ES cells were injected into the blastocoel cavity of an early embryo. Embryos were then surgically transferred to pseudopregnant female mice. Chimeric animals from this litter were backcrossed at maturity, until entirely *Gclm* null animals were recovered. (The above described methods are standard procedure in the production of knockout animals.) The β -galactosidase domain of β -Geo is a reported gene in null and hemizygous animals, as it is under transcriptional control of the *Gclm* native promoter. As such, enzymatic activity of β -galactosidase allows *in situ* proxy determination *Gclm* promoter activity among these animals.

Eight breeding pairs were established. Four pairs consisted of a hemizygous (+/-) male bred to a null (-/-) female, while the remaining four pairs consisted of a hemizygous male bred to a hemizygous female (Table 1). These breeding pairs were chosen with the goal of producing offspring representing the three possible genotypes for *Gclm* –

wildtype, hemizygous, and null. All animals were housed in plastic shoebox cages, in a specific pathogen-free (SPF) facility, accessible only by SPF-trained individuals.

Table 1. Breeding Pairs. The table shows the eight breeding pairs established.

Males¹			cross²	Females³			Treatment⁴
genotype	ID	DOB		genotype	ID	DOB	
+/-	44108	1/13/'03	X	-/-	44696	3/14/'03	MeHg
+/-	42900	12/14/'02	X	-/-	44105	1/3/'03	control
+/-	42821	12/8/'02	X	-/-	42894	12/14/'02	control
+/-	42819	12/8/'02	X	-/-	42870	12/10/'02	MeHg
+/-	42871	12/10/'02	X	+/-	44697	3/14/'03	control
+/-	42824	12/8/'02	X	+/-	44625	3/4/'03	MeHg
+/-	42822	12/8/'02	X	+/-	44626	3/4/'03	control
+/-	42823	12/8/'02	X	+/-	44627	3/4/'03	MeHg

¹Three columns provide data on males, including *Gclm* genotype, assigned University of Washington identification number, and date of birth.

²Column denotes a cross to the female in the corresponding row.

³ Three columns provide data similar to ¹, concerning females.

⁴ Conveys treatment status of the female.

Animals were maintained on a diet of mouse chow, on a 12-hour light-dark cycle.

All practices were accordant with University of Washington IACUC Approved Animal Use Policies.

The morning after the pairs were established, females were examined for copulatory plugs, and the date was designated gestational day (gd) 0. On gd 14, 4 females were administered 6mg/kg mercury as MeHg hydroxide in sesame oil (Alfa Aesar, Ward Hill, MA) via intraperitoneal (i.p.) injection. The remaining 4 females received the sesame oil vehicle via i.p. injection, as a negative control. Females were observed over the following 48-hour period for signs of toxicity associated with treatment.

Tissue collection

On gd 17, the females were sacrificed by CO₂ narcosis, followed by cervical dislocation (males were similarly euthanized prior to gd 17). Maternal brain, liver, and kidney, as well as placental and yolk sac tissue, were dissected, wrapped in aluminum foil, and snap frozen on N₂ (liq.), then transferred to –80°C for storage until analysis. Maternal tissue was identified by the female's identification number. Embryonic tissue (including placenta and yolk sac) was identified using the maternal identification number, followed by a letter and number indicating orientation of the pup (e.g. R2, referring to the second pup, anatomical right). Tissues were later halved on dry ice for protein and RNA analyses. Intact embryos were dissected from females, stored in ethanol overnight, then fixed in paraformaldehyde. Embryos were oriented properly in cassettes and sent to the University of Washington histology facilities for embedding in paraffin wax blocks.

Genotyping

Paraffin-embedded embryos were sagittally sectioned by Leica microtome, with an average section thickness of 16 μm (Leica, Bannockburn, IL). Embryonic tissue sections were deparaffinized with xylenes, and digested at 55° C overnight in Qiagen Buffer ATL plus proteinase K, to lyse the tissue and degrade protein (Qiagen, Valencia,

CA). DNA was isolated from these lysates using Qiagen DNeasy kit, as per directions for rodent tails (Qiagen, Valencia, CA). Isolated DNA was used as a template for polymerase chain reaction (PCR). To determine the embryos genotypes, PCR was used to amplify sequences specific to either the first exon of the *Gclm* wildtype gene or that of the knockout gene. The same forward primer, corresponding to a sequence upstream of the first exon, was used to amplify both genes (Invitrogen, Carlsbad, CA). Reverse primers were complementary to sequences found in either the first exon of *Gclm* or the β -Geo coding region (Invitrogen, Carlsbad, CA). PCR was performed for both the wild type and knockout genes (annealing at 60°C, for 34 cycles) with each embryonic tissue sample, and the product was run on a 1% agarose TAE gel. Embryo genotype was determined by presence or absence of PCR product for the amplified gene. Presence of PCR product for both amplifications in a single tissue signified the tissue derived from a hemizygote, whereas as PCR product for only one of the amplifications signified homozygosity for that gene.

Western Blotting

Tissues were placed on ice in 300 μ l TES-SB buffer plus Roche miniComplete tablet protease inhibitor, and homogenized using a Polytron homogenizing probe (Roche Applied Science, Indianapolis, IN). Homogenates were spun down for 20 minutes at 10,500 rpm and 4°C. Clarified supernatant was recovered and kept on ice. Plate reader-based protein assays were conducted with Bio-Rad protein assay dye, using the Bradford

method to determine protein concentration (Bio-Rad, Hercules, CA). 25µg protein samples were separated electrophoretically on Bio-Rad Ready Gels 12% Tris-HCl and transferred to PVDF membranes (Biorad, Hercules, CA; Millipore, Bedford, MA). Membranes were probed with rabbit polyclonal antisera raised against ovalbumin conjugates of GCLC and GCLM peptides (1:20,000 dilution) overnight, followed by secondary probe with HRP-conjugated goat anti-rabbit IgG antibody (1:50,000 dilution) incubated for 2 hours. Detection was performed with ECL Western Blotting Detection Reagent (Amersham, Piscataway, NJ). Densitometric analyses were performed on Western blot bands, using the Bio-Rad Gel-Doc system (Bio-Rad, Hercules, CA).

Reverse Transcription-Polymerase Chain Reaction

RNA was isolated using the Trizol method. Tissues were placed in Trizol (1ml/100mg tissue) on ice, and homogenized using a Polytron homogenizing probe. Chloroform was added to each sample at a 1:5 ratio with the Trizol. The sample was shaken, incubated at room temperature for 3 minutes, then centrifuged at 12,000 rpm for 15 minutes at 4°C. Aqueous phase material was recovered and mixed with an equal volume of isopropanol, and incubated at room temperature for 10 minutes. After a second centrifugation, supernatant was removed and the remaining pellet was air-dried. The pellet was resuspended in DEPC-treated deionized water and incubated at 55°C for 10 minutes. RNA concentration and purity were measured by absorbance at 260 and 280 nm by spectrophotometer. Small-scale RNA isolation was conducted with yolk sac tissue

using Ambion's RNAqueous-Micro kit (Ambion, Austin, TX). cDNA was reverse transcribed from 2 μ g RNA samples, after DNase treatment, and random nine-mer priming. First strand synthesis was performed at 45°C for 120 minutes, with 2 μ g samples in the presence of 10mM dNTP mix (Roche Applied Science, Indianapolis, IN), 1X first strand buffer, 100 mM DTT, and 100 units SuperScript II enzyme (Invitrogen, Carlsbad, CA). Synthesized cDNA was kept at -20°C until delivery to the Biomarker lab at the University of Washington, for PCR amplification of *Gclm* and β -Geo sequences.

β -Galactosidase activity

Paraffin-embedded embryos were sagittally sectioned by Leica microtome, with an average section thickness of 16 μ m (Leica, Bannockburn, IL). Sections were mounted on glass slides. After deparaffinizing mounted tissues, staining for β -galactosidase activity was conducted using X-gal substrate (5-bromo-4-chloro-3-indolyl- β -D-galactoside). This substrate is cleaved by the enzyme to produce units which dimerize, forming a stable, blue product. Stained sections were visualized using light microscopy, to localize β -galactosidase activity within the embryo, as a proxy for localization of *Gclm* promoter activity. For more sensitive β -galactoside activity measurements, Roche's β -Gal Chemiluminescent Reporter Gene Assay was employed using total protein isolates from deparaffinized embryonic tissue (Roche Applied Science, Indianapolis, IN).

Results

On the morning after breeding pairs were established, no copulatory plugs could be distinctly identified. Often, copulatory plugs only last for 12 hours after mating, and dissolve upon disruption. Because they are not easily detectable, nor does their presence ensure conception, the experiment was continued in the hopes that conception had occurred. Upon sacrifice of the females on gd 17, it was observed that only three of the eight females had successfully conceived, producing a total of 17 pups (Table 2). Female 44696 produced a litter of 8 pups, 6 on the right and 2 on the left. Female 42894 produced a litter of 4 pups, 2 on the right and 2 on the left. Female 44870 produced a litter of 5 pups, 2 on the right and 3 on the left.

The three females were all *Gclm* null (-/-). Because all breeding males were hemizygous (+/-), offspring produced from the crosses were either hemizygous (+/-, 11 pups) or null (-/-, 6 pups); no homozygous wild type (+/+) animals were produced. Genotyping of the embryos supported this conclusion. Figure 5 shows two examples of the genotyping gels, with the PCR products for both the wildtype and knockout sequences. A summary of pup data, including genotype and treatment group can be seen in Table 3. Of the 11 hemizygous pups, only 1 was untreated (42894 L2). The other 10 were treated with MeHg.

Table 2. Breeding Results. The table shows breeding pair data given in Table 1, plus results of the crosses. Data given in red indicate females which conceived.

Males			cross	Females			Treatment	Pups ¹
genotype	ID	DOB		genotype	ID	DOB		
+/-	44108	1/13/'03	X	-/-	44696	3/14/'03	MeHg	8
+/-	42900	12/14/'02	X	-/-	44105	1/3/'03	control	
+/-	42821	12/8/'02	X	-/-	42894	12/14/'02	control	4
+/-	42819	12/8/'02	X	-/-	42870	12/10/'02	MeHg	5
+/-	42871	12/10/'02	X	+/-	44697	3/14/'03	control	
+/-	42824	12/8/'02	X	+/-	44625	3/4/'03	MeHg	
+/-	42822	12/8/'02	X	+/-	44626	3/4/'03	control	
+/-	42823	12/8/'02	X	+/-	44627	3/4/'03	MeHg	

¹Number of pups produced per respective litter.

Table 3. Pup Data. The table shows the results of the breeding pairs. The three litters produced from the eight crosses are shown. Note that two of the three litters received MeHg treatment, and are separated from one another by a dashed line.

maternal ID ¹	pup #	MeHg ²	Genotype ³	
			(+/-)	(-/-)
44696	R1	Yes		X
	R2		X	
	R3		X	
	R4			X
	R5		X	
	R6			X
42870	L1	YES	X	
	L2		X	
	R1		X	
	R2		X	
	L3		X	
42894	R1	NO		X
	R2			X
	L1			X
	L2		X	

¹ The first column gives the mother's identification number. The following column gives the pup's identification, based on location of pup's development in the mother.

²Indicates treatment

³ Two columns show the two possible *Gclm* genotypes; an X indicates the genotype given at the top of the column. The red X denotes the single untreated hemizygous pup.

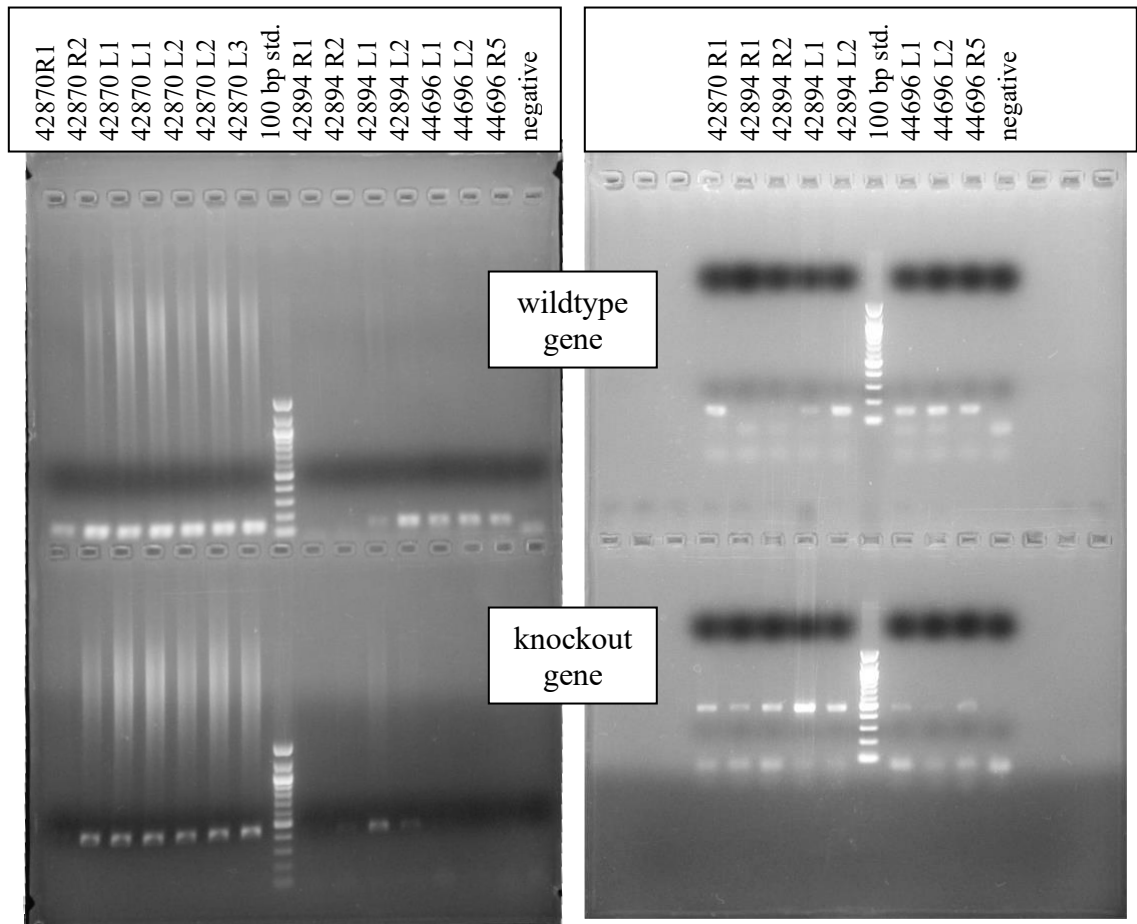


Figure 5. Example Gels Showing Genotyping PCR Products. The 1% agarose gels show electrophoresed PCR products for wildtype and knockout gene amplifications, stained with ethidium bromide and visualized by ultraviolet light. The gel on the right was performed to re-check the genotype of samples run in the gel on the left or in a previous genotyping gel, not pictured in this text (multiple “re-checks” were performed and results were not shown). Because all pups were either hemizygotes or knockouts, each sample is expected to produce a band for the knockout gene amplification, seen in the lower half of the gels.



QuickTime™ and a TIFF (Uncompressed) decompressor are needed to see this picture.

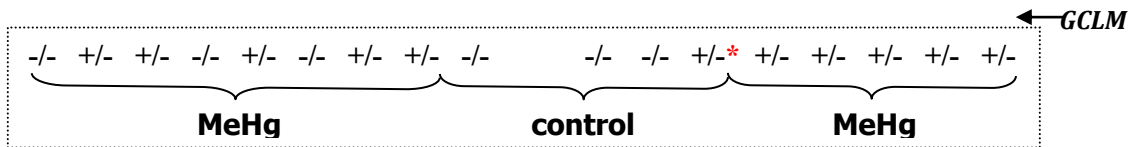


Figure 6. Western Blot for GCLM on Placental Protein. The 27 kDa GCLM protein can be seen just above the 25 kDa line given by the protein standard in the far right lane (obscured by arrow).

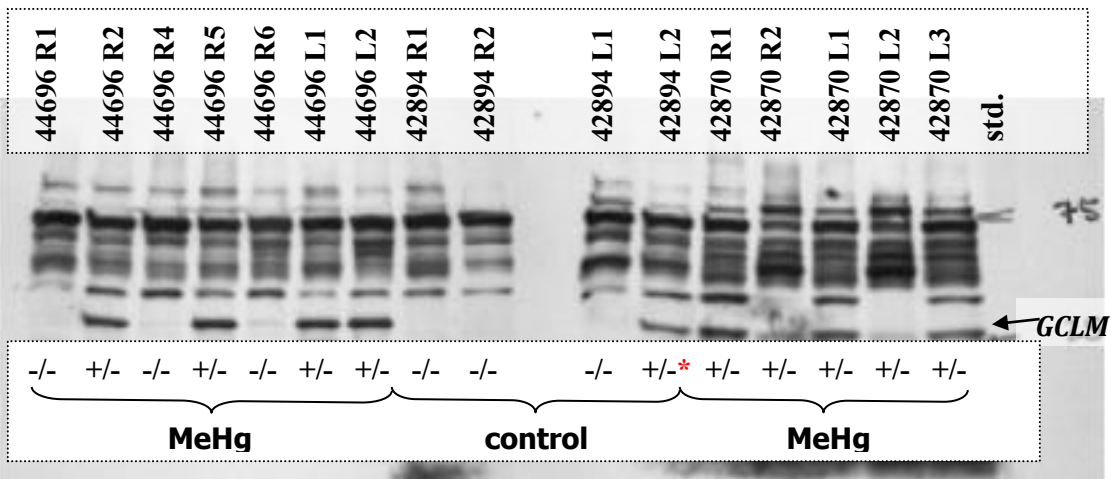


Figure 7. Western Blot for GCLM on Yolk Sac Protein. The 27 kDa GCLM protein can be seen just above the 25 kDa line given by the protein standard in the far right lane (partially obscured by arrow). Note that sample 44696 R3 is missing. The red asterisk denotes the single, untreated hemizygous sample.

Protein expression patterns observed in GCLM-probed Western blots reflected tissue genotype – but not treatment group -- and supported the conclusions of the genotyping assays (Figures 6 and 7). It should be noted that detection of GCLM protein occurred in some of the knockout animal protein samples (*vide infra*, Discussion).

Yields of RNA from yolk sac tissue were very low. This required that samples be concentrated before RT-PCR was performed. Concentration of the RNA was performed by technicians within the Biomarker lab at the University of Washington, who then used concentrated samples to perform RT-PCR, as described below for placental RNA.

The Biomarker lab was also supplied with cDNA, reverse transcribed from placental RNA. Using this cDNA as a template, quantitative PCR was performed to measure mRNA transcripts of *Gclm* among samples. At the time of this printing, however, no RT-PCR data for placental and yolk sac tissue were yet available from the Biomarker lab.

Mounted embryo sections were incubated with X-gal substrate to localize β -galactosidase activity through a cross-section of the embryo. Staining efforts with X-gal failed, however, because enzymatic activity was too low for adequate X-gal cleavage and visible product formation.

Upon macroscopic examination, no gross abnormalities could be recognized among the pups, with the exception of one pup, which had experienced a brain hemorrhage. This pup, 44894 R2, was a null mouse, but was not from a litter which received prenatal MeHg exposure (Tables 2 and 3).

Discussion

Assessment of Results

In undertaking these research efforts, it was hoped that a greater understanding could be gained of GCLM's role in MeHg-induced developmental toxicity. It was hypothesized that *Gclm* null mice would exhibit compromised ability to cope with oxidative stress, and would therefore exhibit greater toxicity than wildtype or hemizygous mice, in response to MeHg exposures. This reduced capacity for coping with the pressures exerted by MeHg, was expected to manifest itself as morphological abnormalities in the developing animal. The only congenital abnormality detected among the pups, however, was a brain hemorrhage in one untreated animal.

Prenatal MeHg exposures have repeatedly been shown to produce gross deformities in offspring. Thus, while the lack of gross deformities observed among treated knockout animals might suggest that GCLM is not involved in the development of MeHg toxicity, it may have, alternatively, been too low a dose to produce immediately evident deformities in either group. Had deformities been comparable and readily apparent in both treated knockout animals and hemizygotes, the hypothesis that GCLM is involved in MeHg toxicity could more easily have been tested.

It was further hypothesized that the *Gclm* promoter was responsive to MeHg exposure, and therefore higher GCLM protein levels would be observed among treated hemizygous pups than untreated hemizygotes. It was hoped that a comparison between the two groups of hemizygous pups would provide data concerning the effect of MeHg

exposure on GCLM protein expression. The Western blots illustrate, however, that no correlation could be seen between MeHg exposure and increased GCLM protein (Figures 6 and 7). (GCLM protein detection among some knockout animals was initially believed to be due to maternal protein contribution, but as all females which conceived were *Gclm* null, it was later thought most likely to be carry-over from adjacent wells, containing protein samples from hemizygous animals.) Based on densitometric analyses, the protein expression datum provided by pup 42894 L2 could not support the hypothesis that MeHg-exposure upregulated GCLM expression, because the density value for the 42894 L2 band fell within the range of density values for the MeHg-treated (+/-) group (note that all samples were standardized at 25 μ g total protein). Additionally, no statistical analyses could be performed concerning this datum, as no standard error could be derived given N=1. It was additionally hypothesized that GCLM transcription and translation would be greater in wildtype animals of a particular treatment group than hemizygous animals of the same group. This hypothesis could not be tested, however, as no wildtype animals were produced from the crosses. Thus, the primary source of difficulties in testing all hypotheses was an inadequate sample size.

As the β -galactosidase reporter gene was under transcriptional control of the *Gclm* native promoter, staining of sagittal sections could act as a proxy for localization of *Gclm* promoter activity in null and hemizygous animals. Consequently, it was hypothesized that MeHg-treated *Gclm* knockout animals would exhibit higher β -galactosidase activity than untreated knockouts. Furthermore, the expression pattern of β -galactosidase in these treated knockout animals was predicted to parallel that of *Gclm* in MeHg-treated wildtype mice. As previously described, efforts to localize β -galactosidase in this manner failed.

This technique was recognized by laboratory technicians to likely have failed due to tissue treatment, as the animals had been observed to express β -galactosidase even without treatment. Activity levels might have been in a detectable range shortly after the embedding of the tissue, but samples were stored at room temperature for some time before sectioning and staining. The processes of tissue fixation and paraffin-embedding, combined with room-temperature storage, likely led to protein cross-linking and reduced activity of β -galactosidase in the tissues, rendering this particular assay ineffective as a test.

Localization of β -galactosidase might have proved more successful if incubation with X-gal substrate had occurred very shortly after paraffin-embedding, and if the incubation was extended to 24-48 hours. Alternatively, localization might have been performed by immunohistochemistry, using labeled anti- β -galactosidase antibodies. This technique, however, might not yield results if the fusion protein no longer exhibits the epitope to which the antibody is reactive. Alternatively, *in situ* hybridization for *lac Z* or *Gclm* mRNA transcripts could be utilized for localization studies. This technique might be more likely to yield results, with the stipulation that embedded tissues be stored at -80, in order to minimize degradation of mRNA. In terms of assays for actual activity of β -galactosidase, there is an alternative to X-gal staining, which employs chemiluminescence measured by plate-reader. While this assay is extremely sensitive to enzymatic activity -- because of the plate-reader's ability to detect extremely low levels of photon emission -- it does not allow tissue localization of activity.

The design of future studies will have to take into account some of the difficulties described here, in order to derive meaningful data. Of primary consideration in the

design of further studies would be the number of breeding pairs established. This figure would ultimately need to be statistically determined, based on knowledge of fecundity rates for this particular population of mice, in order to derive a number of offspring suitable for the desired assays. Furthermore, efforts would need to be made to produce pups representing all three genotypes, such as exclusive use of hemizygotes in breeding pairs.

In addition to GCLM protein and mRNA presence, examination of tissues for mercury speciation might be of interest. Such data might provide more evidence as to how mercury is stored and metabolized in the body, and how this might govern its toxicity. A further point of study might involve a more close examination of maternal tissues. A comparison of maternal tissue mercury levels between MeHg-treated females which had conceived and those which had not, might provide insight about how the developing fetus may be used to reduce the maternal body burden.

Concluding Remarks

It has become increasingly apparent that the MeHg found in many fish is readily passed from the mother's body to the developing fetus. In light of this, the demand for research efforts to address the mechanisms underlying its embryotoxicity has grown. Evidence from past epidemics of MeHg poisoning has suggested that MeHg toxicity may be incurred by the offspring of seemingly healthy women. Thus, it has been recognized that MeHg-induced developmental toxicity may not be foreseeable. In such cases, the

genotypes of the mother and developing child may be determinants in the extent of toxicity and the degree of impaired development.

In this study the role of GSH synthesis in MeHg-induced embryotoxicity was assessed through experiments involving *Gclm* knockout mice. Polymorphisms in *Gclm*, or other genes involved in the GSH antioxidant system, may ultimately prove to be primary determinants of toxic endpoints. While the data collected have not provided a definitive explanation of the role of GCLM in MeHg-induced toxicity, they may provide the bases for future studies. Many possible assays not performed here (described in *Assessment of Results*) could be utilized in future work, in order to provide additional sorts of useful data.

While such animal studies do provide important data concerning MeHg toxicity in mammals, studies on human populations are of infinite value. For example, more data need to be gathered on the extent of polymorphisms in the general population for both the coding regions for the functional domains of *Gclm*, and for regulatory regions of *Gclm*. These data would improve epidemiologic studies and help determine correlations between genetic variation and variation in toxic response. It would be particularly interesting to look at such polymorphisms from preserved tissue of victims of both the Minamata and Iraqi tragedies, and compare them to the extent of toxicity suffered by those individuals.

In conclusion, further studies need to be conducted, taking into consideration both the increasing rates of exposure seen today, and the role of gene-environment interactions. As the body of genetic and genomic knowledge grows, so too does the ability of the researcher to identify links between environmental and genetic factors.

With more work, risk factors as easily identified as polymorphisms, might be implicated. If this were proven true, prospective parents could undergo simple, confidential genetic screening, in a prophylactic effort to reduce toxicity risk to the developing child. These screenings could be followed by dietary and exposure counseling, based on genetically-determined risk. Not only could these efforts reduce annual rates of birth defects in this country, they could also reduce national economic strains resulting from the annual medical costs of caring for and treating birth defects.

It is the hope of this researcher, then, that studies such as the one described here will eventually result in public health strategies to reduce the occurrence and extent of MeHg-induced developmental toxicity. Eventually, this work might elucidate some of the mysteries surrounding human development, and utilize that knowledge in order to reduce perturbations to that development.

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