

**EXAMINATION OF DIOXIN AND ITS ALTERATION OF
GENE EXPRESSION VIA DNA MICROARRAY ANALYSIS**

An Honors Thesis

by

JUSTIN CHARLES WRIGHT

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A & M University
in partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2004

Major Subject: Biomedical Science

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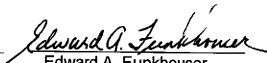
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April 2004

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ABSTRACT**Examination of Dioxin and Its Alteration of
Gene expression via DNA Microarray Analysis.**

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Endocrine disruptive chemicals are known to produce harmful developmental effects on humans and other animals. Since substantial quantities of these chemicals are concentrated in the fat reserves of their victims, it is reasonable to expect a correlation between chemical concentration and physical effect before and to research further into the actuality of the EDCs effects. Some health hazards that are suspected to result from chemical exposure in humans are cleft lip and palate problems, feminization of male offspring, extreme premature puberty in female offspring, neural tube defects, autism, ototoxicity, fetal alcohol syndrome (FAS), fetal tobacco syndrome (FTS), Type II diabetes also known as Non Insulin Dependent Diabetes Mellitus (NIDDM), and ADD/ADHD.

Little evidence has been available to demonstrate how dioxin specifically alters gene expression, both in developing embryos and adults. Recently, Texas A&M University has acquired several DNA Microarray Systems, which are revolutionizing the examination of research into gene expression alterations.

A new cell line of human embryonic kidney cells (293T/17 epithelial) have been properly cultured, had the RNA successfully isolated, and patterns were interpreted for genetic change using DNA Microarray Analysis. Through a cDNA slide specifically spotted with DNA of approximately 1200 endocrine regulated genes, the RNA of these cells can be examined using the DNA Microarray System after being exposed to different concentrations of dioxin, estradiol, DMSO, combinations of chemicals, and finally a control line of unexposed cells. Specific altered genes of the human embryo are predicted to be represented as

changed in the RNA of cells previously exposed to dioxin. A nearly undetectable amount of dioxin (10^{-4} M) was introduced to these cells and produced significant variation from the natural gene expression. These data suggest that major advances in the prevention of physical pain and deformities in both developmental and everyday lives of both humans and animals could be attained by reducing exposure to environmental chemicals. The genes that are altered by the effects of TCDD promise further research, investigation, and prevention of many disruptive diseases.

DEDICATION

This research is dedicated to the many children and adults who have suffered from the effects of dioxin. It is also dedicated to John, Ann, and Cason Wright for their various methods of support and for simply listening to my brainstorming for countless hours. This also is dedicated to the people who have researched in depth before me. Without their shoulders to stand on, I may have never accomplished this research.

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TABLE OF CONTENTS

	Page
ABSTRACT.....	iii
DEDICATION.....	v
ACKNOWLEDGEMENTS.....	vi
TABLE OF CONTENTS.....	vii
INTRODUCTION.....	1
OBJECTIVE.....	1
TCDD PROPERTIES.....	3
PROBLEMS WITH TCDD.....	4
BIOACCUMULATION IN FAT.....	5
SOURCES	
Common Exposure Sources.....	5
Combustion.....	6
Chemical Manufacturing.....	6
Power / Energy Generation.....	7
Metal Smelting Refining.....	7
Reservoirs.....	8
CURRENT DIOXIN REGULATIONS IN THE U.S.	8
TOXICITY	
Acute.....	8
Chronic.....	9
METHODOLOGY AND PROCEDURES	9
Cell Line.....	9
Safety Level.....	10
Thawing Cells.....	11
Feeding Cells.....	11
Subculturing.....	12
Freezing Cells.....	13
RNA Isolation.....	13

SUMMARY AND CONCLUSIONS

Conclusions.....	15
Summary.....	16

REFERENCES.....	18
-----------------	----

VITA.....	27
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INTRODUCTION

Over the past two decades, research has proven that endocrine disruptive chemicals (EDCs) such as the families of the polychlorinated biphenyls (PCBs), organochlorines (OCs), and substituted aromatic hydrocarbons (AHs) are becoming more prevalent as environmental pollutants. Even the most minute concentrations of EDCs are capable of changing both human and animal gene expression. Furthermore, other combinable chemical factors can make the determination of exactly *how* a particular gene is being altered quite difficult to discern (1). Normally chemicals are labeled endocrine disruptive chemicals (EDCs) because of their ability to bind to cellular receptors for steroid hormones. It is imperative for the proper hormone to bind to its designated receptor since the physiological development of both the fetal individual and the adult depend on it. Gene expression is directly correlated with the binding of hormones to their specific receptors (2-6, 18). People and animals worldwide are exposed to EDCs from many sources, which have the potential to bind to steroid receptors and thus skew the hormone-regulated process and thus alter gene expression.

OBJECTIVE

The goal of this research is to determine which genes, and to what degree these genes, are altered when they are exposed to TCDD (2,3,7,8 tetrachlorodibenzo-

The format is the in the style of the Journal of Endocrinology

p-dioxin), more commonly known as dioxin. TCDD may be responsible for birth defects such as neural tube defects, autism, ototoxicity (toxicity to nerve supply of ear), feminization of male offspring, morphological abnormalities, precocious puberty (abnormal timing of pubescent stages), cleft palate, diabetes type II, possibly attention deficit disorder (ADD) / attention deficit hyperactive disorder (ADHD), and finally a decreased immune system function which this paper will emphasize (7, 8, 9-18).

Aim 1: Learn to culture cells effectively and efficiently while watching over the safety of both the cells and personnel involved.

Aim 2: Completely observe the analysis of the environmental contaminant dioxin using DNA microarray analysis.

Aim 3: Determine degrees of alteration of gene expression by dioxin that is significant enough to alter the physiology of living cells.

Aim 4: Derive from the data exactly which genes are altered most significantly with a focus on genes involved in the development of human embryos and immune system function.

Hypothesis 1: Since TCDD is found where so many birth defects are taking place, the microarray will provide a much better understanding of the direct physiological changes that this chemical has on human life *in vivo*.

Hypothesis 2: Human fetal cells exposed to dioxin will show a marked difference in gene expression *in vitro*.

Hypothesis 3: Through evaluation using the array analyses, chemicals can be identified and reduced, then decrease, and thereafter will be prevented from further altering the lives of people.

TCDD PROPERTIES

Commonly called by the trade names dioxin and tetradoxin, TCDD ($C_{12}H_4Cl_4O_2$ MW: 322) has a very simple planar structure, a normal white to colorless crystalline needle-like appearance, a melting point of 305-306°C, and has been labeled as a very toxic and dangerous chemical by the EPA. Dioxin is soluble in organic solvents, mildly in n-octanol, methanol, and lard oil, and insoluble in water. However, because of its extremely balanced structure it remains stable in water, acetone, 95% ethanol, and DMSO. Although TCDD will not ignite, when heated to 500°C or left under UV radiation, it will spontaneously degrade and thus its toxicity is abrogated (19, 20, 21). Except for unique cases such as natural fire from lightning, volcanoes, or seeping fossil fuels, dioxin for all

practical purposes is not produced in large quantities naturally in the environment. The United States of America does not allow commercial production of TCDD within its borders but does allow laboratory production for experimental purposes only (21). Exposure to *any* amount of TCDD may lead to immediate toxicity and / or long-term detrimental defects (35).

PROBLEMS WITH TCDD

Historically, TCDD has been *suspected* to be a carcinogen. In January of 2001, its classification by the EPA changed to a *known* carcinogen because of in-depth research and studies completed between TCDD exposure and neoplasm in humans. Not only has evidence of TCDD causing cancer in humans been shown but also in many different animals. Multiple species exposed can have both malignant and benign neoplasms in various sites and tissues throughout their body. Both sexes of mice, rats, and hamsters exposed to different amounts of TCDD in different ways have produced tumors: in the skin, gastrointestinal tract, and intraperitoneal area. These symptoms increase as the amount of exposure increases. Cancer in the hepatobiliary, lymphatic, adrenal cortex, nasal turbinates, skin, tongue, thyroid, and respiratory systems frequently occur after exposure to TCDD (22, 23).

BIOACCUMULATION IN FAT

Because of its relatively uniform structure and hydrophobicity (not water soluble), TCDD accumulates easily in fat, and is extremely resistant to biodegradation. Studies show that the biological half-life of TCDD in the fat of rodents is from 10 to 30 days while human TCDD retention has a half-life of 5.8 to 11.3 years, presenting much more long-term exposure in humans. This simply means that being exposed at a chronic low-dose, human tissue accumulates TCDD much faster versus lab animals. It also implies that more genetic alteration would be expected in humans following extended exposure as compared to any experimental animal (24). TCDD has been found in the fat reserves of mammals exposed to pesticides, paper mills, effluents, diesel fumes, some herbicides, plasticizers, and bi-products of other chemical manufacturing. Dioxin bioaccumulates rapidly and metabolizes slowly, probably altering the gene expression of exposed mammals (25-27).

SOURCES

Common Exposure Sources

TCDD can be exposed to the general public through ingestion, inhalation, and dermal contact. Dairy, meat, and fish products are important sources of dioxin exposure. Some common sources of dioxin are vacuum cleaner dust, car and room air filters, and clothes dryer lint (28).

Combustion

Since TCDD is a by-product of the manufacture of polychlorinated phenols, it has been detected in commercial samples of 2,4,5-trichlorophenol (2,4,5-TCP), pentachlorophenol (a wood preservative), and in the herbicide, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). Incineration of toxic (both industrial boilers and dedicated), municipal, and hospital waste, electrical transformer fires that are filled with PCBs, open burning of domestic waste, biogas combustion, pulp and paper-mill boilers, kraft black liquor recovery boilers, combustion of landfill gas, cement kilns (a. conventional fuels, b. hazardous waste), pulp and paper mill sludge incineration, lime kilns, asphalt mixing kilns, carbon reactivation, forest and brush fires, waste tire combustion, accidental fires, cigarette smoking, crematoria, candles, and kraft mill wood residue boilers all are sources for the by-product TCDD (29)

Chemical Manufacturing

A defoliant in the Vietnam War named Agent Orange contained a significant amount (2-30 ppm) of TCDD. A commonly used herbicide called Silvex, trade name for propionic acid, contains TCDD. When chlorine, chlorine dioxide, elemental chlorine, metal chlorides, sodium hypochlorite, bleaching pulp & paper mills, dioxazine dyes & pigments, alkylamine tetrachlorophenate, pentachlorophenol, phtalocyanine dyes and pigments, mono to tetrachlorophenols (1-4 Cls), chlorobenzenes, ethylene dichloride / vinyl,

chloride monomer (EDC/VCM), chloranil, tetrachlorobisphenol-A, 2,4-D, polyvinyl chloride (PVC), chlorinated pesticides, and printing inks are produced, TCDD will be a by-product (30).

Power / Energy Generation

Another source of dioxin is that of power and energy generation. Oil combustion is responsible for much of its production for industrial, utility, residential, and commercial purposes. Coal combustion, motor vehicle fuel combustion from leaded gasoline, unleaded gasoline, and diesel fuel, and finally wood combustion also contribute largely to the release of 2,3,7,8 TCDD into our environment. It is important to realize that the sources given above are manifested through many forms and fashions.

Metal Smelting / Refining

Still another by-production source is the smelting of primary nonferrous metals, secondary nonferrous metals, magnesium, nickel, aluminum, copper, drum and barrel reclamation furnaces, lead, scrap electric wire recovery, iron and steel production, ore sintering, coke production, electric arc furnaces, and finally ferrous foundries (7).

Reservoirs

Tragically, TCDD has recently been found in sediments, soils, forests, landfills, pentachlorophenol-treated wood, and notably drinking water sources (31).

CURRENT DIOXIN REGULATIONS IN THE U.S.

TCDD is regulated as a toxic pollutant and hazardous waste by The Environmental Protection Agency (EPA) through the Safe Drinking Water Act (SDWA), the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Clean Water Act (CWA), and Toxic Substances Control Act (TSCA) to mention a few. Drinking water has a maximum contaminant level (MCL) set by the EPA at 3×10^{-8} mg/L (32).

TOXICITY

Acute

Much is known at this point about the acutely toxic physiological effects of high levels of EDCs that are found in fungicides, herbicides, pesticides, and some organochlorines (OC), both to humans and to laboratory animals (6-9). At high levels of exposure, an individual can expect to experience weight loss, liver damage, wasting of glands, malfunction of the immune system, loss of reproductive capabilities, increased incidence of cancer, skeletal abnormalities, altered sex determination, going through puberty extremely early or late, brain

defects, and learning disabilities (33-37) Upon exposure to TCDD, common symptoms will include mild to extreme irritation of the respiratory tract, skin, and eyes (10, 38-40, 46).

Chronic

Not much is known about the continual exposure at low levels of EDC. Examples of continual exposure include surface level water sources and water contaminated with pesticides from agricultural pollutants (41). Research has proven that nursing offspring will have extremely high serum levels of EDC in their fat compared to the relatively minute amount that their mothers are exposed to. During their first year of life, infants in the United States will be exposed to 35-53 pg/kg body weight per day of TCDD equivalents through their mother's breast-fed milk (21, 45). The information about what these chemicals alter is extremely important especially since they are being transferred from mother to offspring at such disproportional quantities (36-37). Also proven is that the placental membrane is readily permeable to EDCs which will impact fetal cells exposed to them (42-44).

METHODOLOGY AND PROCEDURES

Cell Line

A human fetal kidney cell line, specifically 293T/17, was purchased from American Type Culture Collection (ATCC). This cell line (Catalog No. CRL-11268) was transformed with adenovirus 5 DNA, was also transfected with SV40

large T antigen. These cells were deposited from Rockefeller University. Two advantages came from this cell line's properties, one being that it is an adherent cell line meaning that it is easily trypsinized, the other being that its morphology is epithelial which has not been thoroughly researched. 293T is very transfectable and comes from the 293 line which had the temperature sensitive SV40 T-antigen gene inserted into it. The seventeenth clone was selected by the depositor for its easy transfectability.

Safety Level

This was a Biosafety Level 2 contaminant. 293T/17 had not been screened for human immunodeficiency viruses, Hepatitis B, or any other such diseases. Because of the risks listed above and to reduce risks of outside RNA, bacterial, fungal, or contamination of any other sort, all personnel wore latex gloves while handling 293T/17, and worked in a biological safety hood, level IIA (NuAire, Plymouth, MN). To further protection of the cell line, the minimum amount of disturbance that took place consisted of moving them from their incubator, to a microscope, and then directly to a UV treated sterile flow hood. Because UV light can alter gene expression, a fluorescent light available in the hood during interaction with the cells.

Thawing Cells

Upon receipt, immediately the cells were brought out of cryopreservation. The vial was thawed in a 37°C water bath for two minutes and then sprayed down with 75% isopropyl alcohol to avoid contamination of the outside of the vial. Media was warmed as well at 37°C for 15 minutes to allow for appropriate pH changes within the solution. The cells were then transferred to a Falcon (Becton Dickinson Franklin Lakes, NJ) 75cm² flask.

Feeding Cells

The 293T/17 cells were routinely observed via naked eye and microscope for opacity indicating bacterial contamination. Dubelco's Modified Eagle's Medium (Sigma St. Louis, MO) with 4mM L-glutamine which requires 4.5g/L glucose, 1.5g/L sodium bicarbonate, and 10% fetal bovine serum (FBS Atlanta Biologicals, Norcross, GA) was warmed in the water bath for 10 minutes while still being sealed for contamination with Para-film (Pechiney Plastic Packaging, Menasha, WI). After correct temperature was achieved (37°C), 75% isopropyl alcohol was applied to the outside of the bottle, the Para-film was removed, and the media was placed into the UV sterilized hood. Old media would be suctioned into a flask with a hose and sterile glass pipette. An electric pipetter, plastic sterile wrapped pipettes, and latex gloves were used for transferring either 10-15 mL of media into the 75cm² flasks or 20-25 mL of media into the 150cm² flasks. The cells were then specifically allowed to grow in a 10% CO₂ air

atmosphere @ 37°C in a humidified environment for anywhere from 1-3 days depending on confluency.

Subculturing

It took 3 days before 90% confluency was reached indicating the correct time for splitting the cell line. Later in the experiment, it decreased into 2 days and then into 1 day. New DMEM, phosphate buffer solution (PBS), and trypsin (AVTG) were warmed to 37°C for 10 minutes in the water bath. Isopropyl alcohol was applied to the outsides of the containers, the para-film was removed, and then was moved to the inside of the hood immediately. Upon splitting, since this is an adhesive cell line, the old media would be suctioned into a flask with a hose and sterile glass pipette. Five milliliters of PBS was added to the T - 75cm² flask and 10 mL was added to the T - 150cm². PBS was then removed immediately. Trypsin was added, 3 mL to the T - 75cm² flask and 5.5 mL to the T - 150cm² which were then placed in the 10% CO₂ air atmosphere at 37°C. Immediately after a period of 5 minutes of incubation, the flasks were placed into the hood and neutralized with 70 mL of DMEM for a 3-way split into the T - 150 containers or 40 mL of DMEM for a 2-way split. For the T - 75 containers, 15 mL of DMEM neutralized and were aliquotted for a 1:2 splitting and 25 mL for a 1:3 splitting. The new passage number, date, cell line, and persons working with the cells that day were recorded on the outside of each flask. Cells were then quickly returned to the same CO₂ regulated incubator for further growth.

Freezing Cells

Dually, as both a safe-guard and as a relief from accumulating too many flasks from subculturing often, a perfectly sound practice is to freeze down cells in a cryopreservation technique. 293T/17 cells had their media removed, were washed with the standard amount of PBS, trypsinized, and then incubated—the middle two at the same times as used for subculturing. After trypsinization, cells were examined to see if they had lifted from their adhesion to the bottom of the flask, and were then transferred into a conical tube for centrifugation at 200X g for 10 minutes. Freezing media comprised of 20% fetal bovine serum (FBS) / 10% dimethyl sulfoxide (DMSO) to complete media. After centrifugation was complete, media was removed and cells were resuspending from the pellet by gently thumping bottom of conical tube. For every 75cm² of cell growth surface in the conical tube, 5 mL of freezing media was added to cells and then aliquotted in 1 mL segments into labeled cryotubes for -80°C freezer preservation. After a minimum of 4 hours in a Cryo Freezing Container by Nalgene, the cryotubes were then transferred to the liquid nitrogen tanks with their location recorded.

RNA Isolation

Isolation of RNA was conducted using the Totally RNA Kit (Ambion, Austin, TX) with a 15cc tube modification. Before beginning work on any samples,

everything was made RNase-free by spraying Ambion's RNA Zap. Samples were then thawed on ice and centrifuged at 4°C. DNase treatment then involved a second kit by Ambion, DNA Free Kit. The RNA concentration was then measured on a BioRad Ultraviolet Spectrophotometer. Then a 1% agarose/formaldehyde gel was used for checking the integrity of the RNA isolated. A first and second strand of complementary DNA was then synthesized. This was completed using the cDNA Synthesis Kit by Roche. After the double stranded cDNA synthesis, the residual RNA was digested. Ds-cDNA was then cleaned of RNase I and Proteinase K by a careful phenol extraction. In order to produce RNA amplification and to label RNA probes, a protocol that generates dye-labeled antisense RNA probes for microarray hybridizations. Dye-containing solutions were protected from light with aluminum foil in order to prevent photo-bleaching. Ambion's Megascript T7 kit and aminoallyl-UTP was used for *in vitro* transcription. A protocol by Frank Wellmer in the Meyerowitz lab at Caltech was used for RNA generation, clean-up, and labeling.

CONCLUSIONS AND SUMMARY

Conclusions

Previously referred to as p27, Cyclin-Dependent Kinase Inhibitor 1B (CDKN1B) which is down-regulated at locus 12p13, is attributed to the loss of heterozygosity (LOH) and also acute leukemia of the lymphoblast. Also labeled KIP1, this gene is responsible for tumor suppression (47). In order for CDKN1B to be activated and for cells to proliferate, cyclins must undergo phosphorylation by CAK. At normal levels of physiological ATP, cell proliferation will proceed normally, but at lower levels, cellular proliferation will cease (48). When there is not a substantial amount of CDKN1B to cooperate with Pten, then the body is much more susceptible to the development of prostate tumors (49, 50). Without CDKN1B, the body will not stop the proliferation of breast cancer cells (51). Neoplastic growth of the pituitary pars intermedia, ovulatory defect, and female sterility may occur should this gene be down-regulated (52).

Studies show that deficiencies in the Kirsten rat sarcoma (KRAS2) often times are associated with colorectal cancer (53). Cells with KRAS2 mutations also had a link between endometrial cancers. Generally, more KRAS2 mutations will occur with aging (54). Previous studies show that cells that did not have substantial quantity of K-Ras could not form xenograft tumors, if they were placed with B-Raf. However, if there was substantial K-Ras, but there were

deletions for B-Raf, cells proved to be highly tumorigenic. This counters the previous idea that KRAS2 is solely responsible for tumor formation (55).

Mutations such as those observed during this study in both of the oncogenes KRAS2 and neuroblastoma rat sarcoma (NRAS) are suspected to be linked to chronic myelomonocytic leukemia (56). Melanomas occur when a cell has deficiencies in NRAS (57).

Another finding is that when retinoblastoma (RB1) is not expressed which is the case of this experiment, many of the head and neck squamous cells will form carcinomas leading to laryngeal cancer (58). When RAC1 is elevated, it will inhibit cell proliferation when needed. Thus it is reasonable to conclude that if RAC1 count is down, then cancers can result because nothing is directing it to terminate (59).

Summary

Endocrine disruptive chemicals are widespread, but as of yet there are neither ways to keep track of the actual exposures to different EDCs nor to be able to see the magnitude of the possible risks of the exposure. Because of the fact that these chemicals are being found in greater amounts in the liver, serum, milk, and fat of humans and animals (33) and also because of the increasing direct correlation of exposure to EDCs and their adverse health effects (34), this

topic has been exceedingly research-worthy. The hope that this research brings is that the culprit chemicals responsible for the increasingly brutal morphological abnormalities will be discovered and dealt with to the benefit and future of human and animal posterity.

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