Mechanistic Studies on the Antagonistic Effect of Chromium on BPDE Mutant Frequency in Human Fibroblasts

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MECHANISTIC STUDIES ON THE ANTAGONISTIC EFFECT OF CHROMIUM ON BPDE MUTANT FREQUENCY IN HUMAN FIBROBLASTS

by

Felicia Codrea

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Felicia Codrea
The purpose of this study was to investigate the possible mechanisms involved in the decrease in mutant frequency when cells are treated with two different types of carcinogens: chromium (VI) in combination with benzo(a)pyrene diol epoxide (BPDE). Three of our working hypotheses are: Cr alone or Cr and BPDE synergistically activate detoxifying enzyme glutathione-S-transferase (GST), Cr alone or Cr and BPDE act synergistically to inhibit cell cycle at the G₁/S boundary thus allowing more time for DNA repair, and Cr alone or Cr and BPDE synergistically activate DNA repair systems which result in more efficient repair of DNA adducts.

The results from experiments measuring GST activity show that the activity of the enzyme is not influenced by the different treatments with carcinogens. The results for the experiments related to cell cycle arrest show that entry into S phase occurs at the same time for all conditions.
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ABBREVIATION LIST

B(a)P- Benzo(a)pyrene
BPDE-Benzo(a)pyrene diolepoxide
BSA- Bovine Serum Albumine
CAK-Cyclin Activating Kinase
CDK-Cyclin Dependent Kinase
CDNB-1-chloro-2,4-dinitrobenzene
DMEM-Dulbecco’s Modified Eagle’s Medium
DMSO-dimethylsulfoxide
DTT-Dithiothreitol
EH-Epoxide Hydrolase
EST-Expressed Sequence Tags
G3PDH-Glucose-3-Phosphate Dehydrogenase
GSH-Glutathione
GST-Glutathione S-transferase
HPRT-Hypoxanthine Phosphorybosyltransferase
MMLV-Moloney Murine Leukemia Virus
PBS-Phosphate Buffer Saline
PVDF-Polyvinylidenefluoride
ROS -Reactive Oxygen Species
SULT-Sulfotransferase
UDPGT-UDP-Glucuronosyl Transferase
CHAPTER I

INTRODUCTION

Cancer and Carcinogenesis

Cancer is a disease in which cells acquire many abnormal characteristics, one of which is uncontrolled growth. In molecular terms, cancer is a disease of abnormal gene expression. Even though this disease has been known for a long time, there is still a great deal to discover. The term cancer is a general one since it is well known that there are many forms in which this disease is manifested. Scientists have observed that there are some chemicals that cause this disease. Those substances are called carcinogens. Historically, one of the first examples of environmental carcinogenesis was reported in 1775 and involved tumor induction in workers exposed to coal tar (Miller et al., 1975).

Carcinogenesis, the transformation of a normal cell to a tumor cell, is a multistep process, usually taking a long period of time (20 years or more) to complete. The human adult is composed of approximately $10^{15}$ cells, many of which must divide and differentiate in order to repopulate organs and tissues. There are very elaborate mechanisms for controlling cell proliferation on one side and cell death, or apoptosis, on the other side. It is clear now that if mutations occur in the genes which control gene proliferation, cancer can be the result (Bertram, 2001).
One primary cause of carcinogenesis is mutation of genes critical for normal cell function. DNA is subject to damage from exogenous agents both chemical and physical. The most frequent chemical reaction giving rise to DNA damage can be characterized as an electrophilic attack upon a nucleophile (Miller et al., 1975). The most frequent base that undergoes this kind of damage is guanine.

It is important to understand that damage to DNA may not be mutagenic itself. Subsequent DNA replication and cell division is often necessary for a mutation to appear. For example, if one carcinogenic molecule binds to a DNA strand at one guanine base, the DNA polymerase will not recognize the complex formed as a normal guanine base during DNA replication. Instead of matching the guanine with a cytosine, the newly synthesized complementary strand may contain a different base (may be any of the four bases A, G, C, or T). Another possibility of damaged DNA is when deletions of some bases occur.

There are many types of carcinogens, and substances from different classes of chemicals cause cellular damage in different ways. It is known that carcinogens, like chemicals found in the environment and radiation, can cause chromosomal mutations leading to abnormal cell function. For example, ionizing radiation can cause direct damage to DNA, including single and double-strand breaks, and also indirect damage as a consequence of radiolysis of water to yield free radicals (Hall et al., 1999). UV light produces DNA damage by a variety of mechanisms, including the formation of oxygen radicals. It can also generate photoaddition products, especially at adjacent pyrimidines. Formation of a cyclobutane pyrimidine dimer including C5 and C6
bond is one of the most common adducts, along with 6-4 photoproducts (Fig. 1) (Mol et al., 1999). Both lesions can cause cellular death by blocking replication or transcription, or induce mutations, which may result in cancer. Sunlight-induced skin cancer from UV exposure is one of the most common forms of cancer.

Fig. 1. Structures of UV Photoproducts.

Another example of environmental carcinogenesis, but this time from a chemical carcinogen, is highlighted in the epidemiological studies of Langard et al.
(Langard et al., 1990). These investigators have shown that industrial workers exposed to Cr (VI) develop more respiratory cancers than the control population.

Chromium Carcinogenesis

Chromium is an essential nutrient for mammalian carbohydrate and lipid metabolism. Vincent reported that there is a strong relationship between glucose metabolism and chromium intake, probably related to insulin action. At a molecular level, a key role is played by the oligopeptide chromodulin (Vincent, 2001). The daily dose of chromium should be between 50 and 200 µg. Some symptoms of insufficient intake of chromium are hypoglycemia, glycosuria, elevated circulating insulin, decreased insulin binding, and increased ocular pressure (Anderson et al., 1997). Nutritional studies showed that trivalent chromium it is not toxic up to 1 mg per day. Cr (III) can be harmful, however. It was reported that upon skin contact, certain Cr (III) compounds can cause irritations and allergies in some sensitive people (Miksche et al., 1997).

It is already well known that some chromium compounds, especially Cr (VI), are human carcinogens (De Flora et al., 1990). The greatest exposure to chromium is occupational, which may take place in the metal refining/chromate industry, metallurgic industry, and also in steel and anticorrosive paint production (U.S. Public Health Service, 1953). Environmental chromium is mobilized through the combustion of fossil fuels, incineration of public garbage, and also contamination of water and land with industrial chromate waste (Burke et al., 1991). The gate of entry of Cr (VI),
as potassium dichromate, into mammalian cells is via general anion transport channels since the chromate anion is very similar in structure to sulfate and phosphate anions. The most affected tissues from chromium (VI) exposure are the skin, the respiratory tract, and the kidneys (Miksche et al., 1997). The effects of chromium (VI) on different organs can be seen in Table 1.

Table 1. Effects of Chromium (VI) on Different Organs.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Effect</th>
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<tr>
<td>Skin</td>
<td>Dermatitis, Sensitization, Chromium ulcers</td>
</tr>
<tr>
<td>Nose</td>
<td>Septal irritation, Septal ulcer, Septum perforation</td>
</tr>
<tr>
<td>Lungs</td>
<td>Irritation, Bronchitis, Carcinoma</td>
</tr>
<tr>
<td>Kidneys</td>
<td>Tubular dysfunction</td>
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</table>

The toxic effect of chromium is due to the reduction of the Cr (VI) ion to the more reactive intermediates Cr (V) and Cr (IV), and to the stable Cr (III), by a spectrum of reductants including glutathione (GSH), cysteine, ascorbate, riboflavin and NADPH-dependent flavoenzymes like microsomal cytochrome P450 reductase (Shi et al., 1999, De Flora et al., 1990, Sugiyama, 1992). Cellular reduction of Cr (VI) is represented in Fig. 2. (Sugiyama, 1991).
The reduced chromium can then increase the concentration of hydroxyl radicals in cells via the Fenton reaction pathway. Cr (V), which is unstable, reacts with hydrogen peroxide as shown below:

\[
\text{Cr}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Cr}^{4+} + \cdot\text{OH} + \text{OH}^{-}
\]

Cr (VI) can be reduced by glutathione and ascorbic acid and generate Cr (IV) which is able to generate ‘OH formation via a Fenton-like reaction (Luo et al., 1996):

\[
\text{Cr}^{4+} + \text{H}_2\text{O}_2 \rightarrow \text{Cr}^{3+} + \cdot\text{OH} + \text{OH}^{-}
\]

The generation of the hydroxyl radical (‘OH) and lipid hydroperoxide derived free radicals from the reaction of Cr (III) with H₂O₂ has also been reported (Shi et al., 1993). The hydroxyl radicals formed are dangerous because they can react with DNA and cause base damage, such as 8-hydroxydeoxyguanosine (Fig. 3), which if left unrepaired can form point mutations (Aiyar et al., 1989). It has also been shown that

Fig. 2. Cellular Reduction of Cr (VI). Modified from Sugiyama, M. (1991).
exposure to chromium can lead to both DNA-protein cross-linking and DNA-DNA cross-linking which can result in mutations in cellular genes (Leibross et al., 1990). Chromium can also induce DNA damage by formation of Cr-DNA adducts (Wu et al., 2001).

Fig. 3. Structure of 8-Hydroxydeoxyguanosine (Wang et al., 1998).

Benzo(a)pyrene Carcinogenesis

Benzo(a)pyrene is a common environmental pollutant that belongs to a class of chemicals called polyaromatic hydrocarbons (PAH). Benzo(a)pyrene can be found in cigarette smoke, fuel exhaust, and any time there is incomplete combustion of organic matter. Benzo(a)pyrene itself is not carcinogenic, but it gets metabolized to benzo(a)pyrene-(7R,8S)-diol (9S,10 R)-epoxide ((+)-anti-BPDE) once it gets into the body. This product is the result of epoxidation, hydration, and further epoxidation of the benzo(a)pyrene (Sanjay et al., 1999). BPDE exists as a pair of diastereoisomers (syn- and anti- BPDE) and each has a pair of optical enantiomers (Fig. 4). The most carcinogenic from of these isomers was found to be the (+) anti-BPDE with a (R, S)-diol (S, R) epoxide configuration. It was shown in previous studies that (+) anti-BPDE induces approximately 22 to 59-fold more mutations than the other three isomers (Hong et al., 1998).
BPDE is carcinogenic due to electrophilic attack on the nucleophilic sites of DNA (and also RNA and proteins), which forms a covalent, bulky carcinogen-DNA adduct. The primary product of the reaction of (+)-anti-BPDE with DNA, is the (+)-trans-anti-benzo(a)pyrene diol epoxide-\(N^2\)-dG adduct (Fig. 5) (Perlow et al., 2001). It is the most mutagenic benzo(a)pyrene adduct in mammalian systems, and causes G-T transversion mutations (Denissenko et al., 1998) because the DNA polymerase does not recognize the bulky adduct as guanine and will mismatch the adduct with adenine during replication. This may happen because the size and shape of adenine opposite
syn (+)-trans-anti benzo(a)pyrene -N²-deoxyguanosine is similar to that of a Watson-Crick pair (Perlow et al., 2001).

Fig. 5. The Structure of the (+)-Trans-Anti-Benzo(a)pyrene Diol Epoxide-N²-dG Adduct (Perlow et al., 2001).

Multiple Carcinogen Treatment

The literature is filled with information about different carcinogens and how exposure can lead to cancer. Most of these studies, however, concentrate on only one substance. In real life, the human body is exposed to a multitude of carcinogens simultaneously. The overall goal of this project is to examine the cellular effect of multiple carcinogen treatments. For this study, we have chosen two different types of carcinogens: chromium (VI) and the polycyclic aromatic hydrocarbon benzo(a)pyrene diolepoxide (BPDE).

Initial experiments were performed by others in the lab to determine the effect of multiple carcinogen treatment on mutant frequencies (Tesfai et al., 1998). Cloning experiments were performed to establish the appropriate concentration of
carcinogens. The cytotoxicity was determined by plating 300 cells/dish in 100 mm tissue culture dishes. Cells were treated for 48 hours with different concentrations (0, 0.01 µM, 0.03 µM, 0.1 µM, 0.3 µM, and 1 µM) of potassium dichromate (K$_2$Cr$_2$O$_7$). The chromium containing medium was then replaced with a medium without chromium and the cells were allowed to grow for two weeks. The dishes were then stained with crystal violet and the number of clones was determined. Triplicate dishes were used for each concentration. Based upon the results of the experiments, and according to the existing literature, 0.3 µM K$_2$Cr$_2$O$_7$ was chosen because it produced approximately a 50% decrease in cloning. Typically, concentrations of carcinogens which show significant decreases in cloning ability are needed in order to observe mutations. BPDE was also tested and a concentration of 0.3 µM and an incubation time of 2 hours was chosen.

Four conditions were established in initial experiments. Cells were treated with either normal growth media for 48 hours (control), media containing 0.3 µM potassium dichromate for 48 hours, normal growth media for 46 hours followed by media containing 0.3 µM BPDE for 2 hours (BPDE), or 46 hours with media containing 0.3 µM potassium dichromate followed by 2 hours with media containing both chromium and BPDE together. Cytotoxicity was determined using the cloning method described above and reported as relative cloning ability with the untreated controls defined as 100%. Results of this part of the experiment are shown in Fig. 6 (Tesfai et al., 1998). When the cells were treated with both carcinogens there was a
decrease in cloning ability of the cells when compared to the results of individual treatments which appeared to be additive.

Fig. 6. Effect of Pretreatment with Cr (VI) Followed by Coincubation with BPDE on Cellular Cloning Ability.

The mutant frequency at the hypoxanthine phosphoribosyltransferase (HPRT) gene was done by incubating cells with the carcinogen(s), as described above, and then allowing the cells to grow in normal growth media without carcinogens for 10 days following treatment. The cells were then replated in 30 µM 6-thioguanine. Normal cells will metabolize this compound into a toxic substance via the enzyme synthesized from the HPRT gene and not grow. If the HPRT gene is mutated, cells will not metabolize the 6-thioguanine and be able to grow. Cells were allowed to
grow in presence of 6-thioguanine for 3 weeks, then dishes were stained with crystal violet and the number of mutant clones counted and corrected for cloning efficiency. When cells were coincubated with both carcinogens, an antagonistic effect on mutant frequency was observed (Fig. 7). It appeared that the chromium treatment protected the cells from the mutagenic effects of the BPDE.

Fig. 7. Effect of Pretreatment with Cr(VI) Followed by Coincubation with Cr (VI) on Mutant Frequency of BPDE.
The question was then raised as to whether the order of adding the two carcinogens influenced the results. Therefore, three different protocols were tested and the results compared to those stated above. The three additional protocols tested were:

1. 48 hours chromium + 2 hours BPDE
2. 2 hours coincubation Cr and BPDE + 46 hours chromium
3. 2 hours BPDE + 48 hours chromium

The results from protocol one showed that chromium had an additive effect on the cytotoxicity of BPDE as described for the previous protocol. When cells were pretreated with chromium before BPDE treatments, the mutant frequency was much higher than when cells were treated only with BPDE. It seemed that the two carcinogens acted synergistically. The results of the second treatment protocol were similar to that obtained with the original treatment schedule (Fig. 6 and Fig. 7). It appeared that chromium protected the cells from the mutagenic effect of BPDE but still maintained the additive effect when cytotoxicity was determined. In the last protocol, the antagonistic effect of chromium on mutant frequency of BPDE was lost with no synergistic effect observed, but the additive effect on cytotoxicity was still present.

The conclusion from this set of experiments is that the order of addition of carcinogens does not affect the results of the cloning ability experiments, but the antagonistic effect of chromium on the HPRT mutant frequency by BPDE was seen only when the two carcinogens were coincubated. This antagonistic effect on mutant frequency seems to be due to the formation of reactive oxygen species (ROS). When
the experiments were performed using vitamin E (antioxidant) in the media, the antagonistic effect of the metal was completed eliminated. The purpose of this study is to elucidate the biochemical mechanism for this antagonistic effect.

Hypotheses

We have proposed the following hypotheses to explain the antagonistic effect on the mutant frequency described above:

1. Chromium alone or chromium and BPDE synergistically activate the enzyme glutathione S-transferase (GST). This enzyme has been shown to inactivate many polyaromatic hydrocarbons by reacting them with glutathione.

2. Chromium alone or chromium and BPDE synergistically halt the cell cycle progression at the G<sub>1</sub>/S boundary and allow the cells more time to repair DNA-BPDE adducts.

3. Chromium alone or chromium and BPDE synergistically activate DNA repair systems which result in more efficient repair of DNA adducts.

BPDE Detoxifying Enzymes

There are many enzymes involved in defense mechanisms to protect cells against toxic compounds. The metabolism of xenobiotic compounds can be characterized as taking place in two distinct phases, commonly referred as phase I and phase II. Usually in phase I a xenobiotic undergoes a transformation, which can be oxidation, reduction, and/or hydrolysis. Oxidation is the dominant reaction and the enzyme which catalyzes this process is cytochrome P450. In phase II, the xenobiotic
or its metabolite is conjugated to an endogenous molecule. These conjugation reactions are catalyzed by different detoxifying enzymes, like epoxide hydrolases (EH) (Siedegård et al., 1997), sulfotransferases (SULT), UDP-glucuronosyl transferases (UDPGT) (Iersel et al., 1999), and glutathione S-transferase (GST) (Hayes et al., 1995). The conjugation products have lower reactivity compared to the toxic compounds and can be excreted from the cell and the body.

Several systems have been developed by mammalian cells that detoxify BPDE. Addition of water to the epoxide moiety of BPDE, to form the corresponding benzo(a)pyrene-7,8,9,10-tetraol, is catalyzed by epoxide hydrolase (Penning, 1993) (Fig. 8). When BPDE is metabolized with the help of sulfotransferase it forms sulfate conjugates (Penning, 1993) (Fig. 9). When BPDE is detoxified by glucuronosyltransferase, the conjugation reaction formation of conjugates between reactive hydroxyl groups and glucuronic acid (Penning, 1993) (Fig. 10).

![Diagram](image_url)

**BPDE**

**B(a)P-7,8,9,10-tetraol**

Fig. 8. Formation of Benzo(a)pyrene-7,8,9,10-tetraol by Addition of Water to BPDE.
The glutathione S-transferases (GST) are a family of multifunctional enzymes important for cellular detoxification (Vanhaecke et al., 1997). They catalyze the conjugation of electrophilic compounds with the glutathione via the thiol group of cysteine (Habig et al., 1974). The conjugation product is exported from the cell via an ATP-dependent efflux pump (Wilce et al., 1994) and then excreted. The cytosolic GST activity of mammalian cells is due to a multitude of isoenzymes designated as alpha, mu, pi, sigma and theta families.
GST also plays an important role in reducing the effects of reactive oxygen species (ROS) and the breakdown products of oxidized DNA (Hayes et al., 1995). Glutathione may play an important role in the protection against oxidative stress induced by metals since it has been shown that Cr (VI) induces high levels of GST (Dubrovskaya et al., 1998, Kitahara et al., 1984). Studies done by Tully and coworkers showed that Cr (VI) produces dose-related two-fold induction of GST Ya in human hepatoma cells HepG2 (Tully et al., 2000). Glutathione S-transferase was also shown to play an important role in eliminating the effect of reactive oxygen species generated by Cr (VI), by reacting the products of ROS with glutathione (Kortenkamp et al., 1996). Reduced glutathione (GSH) manifests a high reactivity towards Cr (VI) (Wiegand et al., 1984) and it was shown that this plays an important role in Cr (VI) metabolism.

It has been demonstrated that purified GST reduces the binding of BPDE to DNA (Hesse et al., 1982). It was also shown that purified human pi-class isoenzymes are more efficient than other classes in binding GSH with (+)-anti-BPDE (Xia et al., 1998). In addition (-)-anti-BPDE is a poor substrate for hGSTP1-1 compared to (+)-anti-BPDE (Fig. 11) (Hu et al., 1997). Fields et al. showed that high levels of expression of h-GSTP1-1 were protective against DNA damage and cytotoxicity induced by BPDE (Fields et al., 1988). Other studies have shown that hGSHTM1-1 the human mu isoenzyme, is believed to play an important role in detoxifying (+)-anti-BPDE (Srivastava et al., 1999). GST is induced by high levels of BPDE (Hayes et al., 1995). Since it was shown that GST is induced by both, chromium (VI) and
BPDE, we chose to test if this enzyme is activated when there is coincubation of the two carcinogens.

Fig. 11. Structure of Anti-BPDE Enantiomers and their Conjugates with GSH. (Hu et al., 1997).
The cell cycle is the period of time from the birth of one cell to the point when the cell divides into two daughter cells. The cell cycle is regulated by a succession of events, and is divided into four phases (Fig. 12):

- G\(_1\) initial growth phase or gap phase
- S synthesis of DNA
- G\(_2\) another gap phase
- M mitosis (cell division) for somatic cells.

After mitosis, cells can enter into a resting state (G\(_0\)), or when receiving appropriate growth signals, enter again into G\(_1\) phase (Tyrcha, 2001). There is another alternative, and that is the cell may receive signals that trigger cell death or apoptosis. The cell cycle is very important because the genetic information is preserved and transferred to the new cells (Dictor et al., 1999). There are several checkpoints in the cell cycle to make sure that the genetic integrity of the genome is preserved. All phases of the cell cycle are subject to checkpoint control. One of the
most important checkpoints occurs in late G₁, about four hours prior to entry into S phase, when the DNA is replicated. Its role is to ensure that the cell does not begin DNA synthesis if the DNA is not integer (Bertram, 2001). If the DNA is damaged, there is a halt at the G₁/S boundary until the DNA repair mechanisms fix the damage or apoptosis is triggered (Sielecki et al., 2000). Once the cell passes the G₁/S checkpoint, it is irreversibly committed to the next division cycle.

One of the key proteins involved in cell cycle progression into the S phase is the retinoblastoma protein pRb. In its unphosphorylated form, pRb binds to E2F (a family of transcription factors) and forms a silencing complex restricting transcription of genes necessary for cell cycle progression (Weintraub et al., 1995). Key enzymes also include cyclin-dependent kinases (CDKs). They are polypeptides that consist of a heterodimer, one catalytic subunit which has no intrinsic activity and one regulatory subunit called cyclin (Hengstschläger et al., 1998). Cyclins are so named because they are synthesized only at a specific time of the cell cycle. The activation of the catalytic subunit occurs in a two-step process (Pavletich, 1999). The first step is association with a cyclin regulatory subunit for activity (Sielecki et al., 2000). This is a very important step because the cyclin levels are strongly regulated by transcription and ubiquitin mediated degradation in a temporal manner (Morgan et al., 1995). The second step is the phosphorylation of the CDK-cyclin complex by the CDK-activating kinase (CAK) (Fisher et al., 1994). The function of the CDK-cyclin complex is to phosphorylate or dephosphorylate specific serine or threonine residues
on target proteins, like retinoblastoma protein, using ATP as a phosphate group source (Sielecki et al., 2000).

There are several cyclins and associated proteins that are involved in the cell cycle and the G1/S transition. Two complexes of interest are formed between cyclin D and CDK 4 and cyclin D and CDK 6 (Fig. 13). Their target is the retinoblastoma pRb protein (Grana et al., 1998, Ekholm et al., 2000). Also cyclin E forms a complex with CDK2, and has a secondary role in phosphorylating the pRb (Draetta, 1994, Harbour et al., 1999). The activity of cyclin dependent kinases is inhibited by the INK family of proteins, which bind to CDKs and inhibit the formation of cyclin-CDK complex. In addition, the p21 family, which contains p21 WAF1/CIP1/CDK1, p27 KIP1, and p57 KIP2, inhibits the cyclin-CDK complex formation (Dictor et al., 1999). An important role in the phosphorylation/dephosphorylation state of pRb is played by the tumor suppressor protein p53. Tumor suppressor genes are genes that control cell growth and can be inactivated by mutations (Bertram, 2001). The p53 pathway is induced by a multitude of factors, like DNA damage and oxidative stress (Liu et al., 2001). DNA damaging agents like γ irradiation, ultraviolet light, or chemical carcinogens can activate p53. This is accomplished by phosphorylation of serine 15 and/or serine 20 of the transactivation domain and both phosphorylation and acetylation of the basic allosteric control region (Giaccia et al., 1998, Lakin et al., 1999, Hickman et al., 2002). The p53 protein activates p21, which inhibits the function of cyclin D: CDK4/CDK6 complex (Fig. 13).
It is reasonable to imply that after carcinogen treatment, the cell cycle will be delayed and the DNA repair mechanisms will be triggered. Xu et al. showed that chromium blocks the cell cycle at the G₁/S boundary (Xu et al., 1996). p53, playing such an important role not only in progression of the cell cycle but also in DNA repair systems (Nagata, 1997, Albrechtsen et al., 1999), may be synthesized in higher concentrations after carcinogenic stress. The p21 protein would then be activated by p53.
CHAPTER II

MATERIALS AND METHODS

Cells and Cell Culture

One cell line used throughout this study was L266-C, a normal human fibroblast cell line derived from human foreskin tissue. It was provided by Dr. Justin McCormick of the Carcinogenesis Laboratory at Michigan State University. Cells with a passage number of 30 or less were used in the reported experiments. The fibrosarcoma cell line HT1080, also obtained from Dr. McCormick, was used for the validation of the glutathione S-transferase assay. Cells were grown in medium composed of Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco/BRL) supplemented with 0.2 mM serine, 0.2 mM aspartate, and 1.0 mM sodium pyruvate and containing 10% supplemented calf serum (SCS) (Summit Biotechnologies), penicillin (100 units/ml), streptomycin (100 µg/ml) and hydrocortisone (10 µg/ml). Cells were incubated in a humidified atmosphere at 37°C with 5% CO₂.

Cell Harvesting and Counting

Cells were washed with phosphate buffer saline (PBS), then treated with a 0.25% trypsin solution in PBS for 4-5 minutes for L266-C cells and 1-2 minutes for HT1080 cells. Cells were washed off plates or flasks using 5 ml of complete medium and counted using a hemocytometer when necessary. The cell counts were determined for plating the appropriate cell density for the incorporation of ³H-thymidine in the DNA labeling experiments, or for normalizing the data for the glutathione S-
transferase activity assay. We could not use the total amount of protein for normalization in the glutathione S-transferase assay due to the fact that BSA was added to each sample.

Chemicals Used

The (+)-r-7,t-8-dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE) was purchased from ChemSym Science Laboratories. A 1.0-mM stock solution of BPDE in DMSO was prepared freshly for each experiment. Potassium dichromate was purchased from Spectrum Chemical Manufacturing and a 1.0 mM stock solution in PBS was prepared, sterile filtered and stored at 4°C. The stock solution of each carcinogen was diluted with the complete medium to the concentration of 0.3 µM for each treatment.

Glutathione S-transferase Assay

Initially, cells were plated onto 150 mm dishes at low density. The cells were allowed to grow, and then treated with or without carcinogens when they had reached approximately 50% confluence. Treatment was done at this time so that we had the maximum number of logarithmically growing cells to assay. After the appropriate incubation period, cells were trypsinized, collected in 5ml PBS containing 1mg/ml BSA and centrifuged at 900 rpm for 3 min at 4°C using a Beckman centrifuge. It was necessary to add BSA to the buffer to help the cells pellet upon centrifugation. The pellets were resuspended in 500 µl PBS and lysed either by sonication or a freeze/thawing method. Before sonication or freeze/thawing, a 50 µl aliquot of each
sample was saved for counting to normalize the results to the number of cells. After sonication, or freeze/thawing, the cell lysate was centrifuged at 14,000 rpm for 40 minutes at 4°C and the supernatant collected.

For the spectrophotometric readings, the instrument was blanked using a solution containing 960 µl 0.2 M phosphate buffer (pH 6.5) and 40 µl 95% ethanol. Next, three readings were taken using only 860 µl phosphate buffer, 100 µl reduced glutathione (10 mM in phosphate buffer, pH 6.5) and 40 µl CDNB (25 mM in ethanol). Since this reaction mixture contains no enzyme, we expect no activity. The average of those three readings was subtracted from the readings of samples containing cell lysates. For each sample, 100 µl of cell lysate was added to a mixture of 760 µl phosphate buffer, 100 µl reduced glutathione (10 mM stock solution) and 40 µl CDNB (25 mM-stock solution) (Dubrovskaya et al., 1998, Hu et al., 1999). The absorbance at 340 nm was then determined for 3 minutes using a Beckman DU-7400 spectrophotometer. Three different readings were taken for each condition, normalized for the number of cells, and expressed as units of enzyme activity per cell, per minute. One unit of activity corresponds to an absorbance change of 3.2 O.D./min and is defined as formation of 1 µmol/min of thioether (Simons et al., 1977).

**DNA Labeling Assay**

Experiments determining cell cycle arrest were performed by measuring the incorporation of ³H-thymidine into DNA. L266-C cells were plated in 100 mm dishes and grown to confluence. After the cells reached confluence they were refed daily with medium containing serum for three additional days, and then not fed for 36 hours
following the final medium change. Cells were designated to be in the \( G_0 \) state at this time. Cells were then treated with or without 0.3 \( \mu \)M \( K_2Cr_2O_7 \), and after 36 hours of treatment, the cells were replated in the appropriate medium (with or without \( K_2Cr_2O_7 \)) at \( 10^4 \) cells/cm\(^2\) in 30 mm dishes (24 dishes for each condition). The cells were incubated for 10 hours, and then the appropriate addition of BPDE and/or Cr (VI) was made (see four conditions in the Introduction). The cells were incubated for an additional 2 hours and then all medium was replaced with fresh medium containing no carcinogens. The first time point was immediately after the carcinogens were taken off (12 hours after release from \( G_0 \)). Every 4 hours, beginning with the initial time point, the media was aspirated off 3 plates and replaced with 1 ml of media per plate containing \( ^3\)H-thymidine (5 \( \mu CI/ml \)). The cells were then incubated for 15 minutes in the presence of the labeled thymidine, washed with PBS, trypsinized, an aliquot of 50 \( \mu l \) counted to determine the number of cells, and the remainder collected on a Whatman filter. The filter was washed twice with hypochloric acid (10%) and twice with 95% ethanol to eliminate unincorporated \( ^3\)H-thymidine. The filter paper was allowed to dry and the radioactivity bound to the filter was determined by liquid scintillation counting (Watanabe et al., 1985). The extent of incorporation was corrected for the number of cells assayed and it was expressed as counts per minute per 100,000 cells.

**Western Blotting Assay**

Lysates from cells treated in various ways were prepared by washing the cells with ice cold PBS and scraping the cells off the dishes in a minimum volume of lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1.0 mM EGTA, 30.0 mM p-
nitrophenylphosphate, 1.0 mM sodium orthovanadate, 50.0 mM NaF, 1.0 mM phenylmethylsulfonyl fluoride, 10.0 µg/ml aprotinin, and 10.0 µg/ml leupeptin). The total protein concentration of the cell lysates was determined using a modified Bradford assay (Bio-Rad Laboratories (CA)), and performed as described by the manufacturer. Protein concentrations were determined so that we could load equal amounts of protein into each well of the gel. Western Blotting was accomplished using a NOVEX apparatus. A 12% acrylamide gel was used. The samples were loaded, the gel was run at 130 V for 75 minutes, followed by transfer of the proteins to a PVDF membrane at 65 V for one hour following the instructions provided by the manufacturer. Membranes were blocked with freshly prepared PBS containing 3% nonfat dry milk (PBS-milk) for 1 hour at room temperature with gentle agitation. The membranes were then incubated overnight at 4°C with 1.0 µg/ml of a mouse monoclonal IgG, a primary antibody (Upstate Biotechnology, NY), directed against p53, diluted in PBS-milk. The blot was washed the next day and then incubated with the anti-IgG secondary antibody (Upstate Biotechnology, NY) linked to horseradish peroxidase (goat anti-mouse IgG), diluted 1:1000, for 1 hour at room temperature. Next, the blot was washed and an enhanced chemiluminiscent (ECL) system (Kirkegaard & Perry Laboratories, MA) was used for detection of proteins. We used Jurkat cell lysate as positive control for p53 (human-T lymphocyte line, Upstate Biotechnology, NY).

We have also tested for the amount of p21<sup>waf1</sup> protein in various samples using the Western Blot technique. The primary antibody was Anti-Cip1/WAF-1/p21 mixed
mouse monoclonal IgGs at 1 µg/ml (Upstate Biotechnology, NY) and the secondary antibody was the same as above. A positive control for p21 was not available.

**cDNA Expression Array**

Cells in the logarithmic phase were treated with the different conditions described in the Results section. Total RNA was isolated from cultured cells using a commercially available kit from CLONTECH, the Atlas Pure total RNA labeling system. The method consists of three rounds of phenol-chloroform extraction, followed by precipitation of the RNA with isopropanol and centrifugation at 12,000 rpm. After centrifugation, the pellet was allowed to dry and resuspended in RNase free water.

The quality of isolated RNA was determined in two ways. First 2 µl of sample was dissolved in 398 µl buffer (10 mM Tris-pH 7.5 and 0.1 mM EDTA-pH 7.5) and the A$_{260}$ and A$_{280}$ were read using a Beckman DU-7400 spectrophotometer. The buffer was used as a reference blank. The A$_{260}$/A$_{280}$ ratio was then determined to ensure it was between 1.9-2.1. The total A$_{260}$ was calculated multiplying the A$_{260}$ reading by the dilution factor (200 in this case). Knowing that one A$_{260}$ unit of RNA is 40 µg/ml, the concentration (µg/ml) of the RNA in each sample was calculated by multiplying the total A$_{260}$ by 40 µg/ml. The RNA yield in milligrams was obtained by multiplying the concentration by the volume of the RNA sample.

The RNA quality was also determined using an agarose gel. Five µg of RNA was loaded onto a 1% agarose gel and run for 1 hour at 75 volts. The gel was washed twice for 15 minutes in DEPC treated water, then the gel was incubated in ethidium
bromide solution (1µg/ml) for 15 minutes followed by washing again twice for 15 minutes in DEPC treated water. Nondegraded RNA appeared as two bright bands (23S and 16S ribosomal RNA) under UV light.

To eliminate genomic DNA, DNase treatment of total RNA (1mg/ml) was done using 10 x DNase buffer and DNase I (lunit/µl). For better results Ambion’s ANTI-RNase (1 unit/µl) was included. For example, 500 µl of a RNA sample (1mg/ml), 100µl of 10 x DNase I Buffer, 50 µl of DNase I (1 unit/µl) and 350 µl of deionized water were added together. After incubation for 30 minutes at 37°C, the reaction was terminated using 100µl 10 x Termination Mix. A phenol: chloroform extraction was done next and then the samples were treated with 1/10 volume of 2M sodium acetate and 2.5 volumes of 95% ethanol. After incubation on ice for 10 minutes, the samples were centrifuged at 14,000 rpm for 15 minutes at 4°C. The pellet was then washed with 80% ethanol, centrifuged at 14,000 rpm for 5 minutes at 4°C, and allowed to dry for about 10 minutes. The precipitate was dissolved in RNase free water and the purity and quality of the RNA checked again, as described above.

The next step was preparation of streptavidin magnetic beads. The magnetic beads (15 µl are sufficient for one sample which contains up to 50 µg total RNA) were washed three times in 150 µl of 1 x Binding Buffer, then resuspended in 15 µl 1 x Binding Buffer. Next, poly A+ enrichment was performed. For every 50 µg of total RNA (diluted up to 45 µl with deionized water) 1 µl biotinylated oligo(dT) was added and the mixture was incubated for 2 minutes at 70°C. The mixture was then cooled at room temperature for 10 minutes followed by addition of 45 µl of 2 x Binding Buffer.
Fifteen µl of the magnetic beads were then added to each RNA sample. The mixture was then vortexed at the highest setting for 25-30 minutes and the beads were separated using a magnetic particle separator. The beads were then washed in 50 µl of 1 x Washing Buffer. The wash was repeated two more times and the magnetic beads were resuspended in 50 µl of 1 x Reaction Buffer. The Reaction Buffer was removed and the beads were resuspended in 6 µl of deionized water.

For cDNA probe synthesis it was necessary to prepare a Master Mix which contained 4µl of 5 x Reaction Buffer, 2 µl of 10 x dNTP mix, 5 µl of $^{32}$P-dATP (3,000 Ci/mmol, 10 µCi/µl) and 0.5 µl of DTT (100 mM) per reaction. One µl of CDS Mix (which contains primers) was then added to the resuspended beads (contain the polyA+ RNA), and the samples were incubated at 65°C for 2 minutes, followed by another 2 minutes incubation at 50°C. Meanwhile, 2 µl of MMLV Reverse Transcriptase was added per reaction to the Master Mix. After the incubation of the beads at 50°C, 13.5 µl of the Master Mix was added to each sample and the reaction mixture incubated for 25 minutes at 50°C. The reaction was terminated by adding 2 µl of Termination Mix.

Purification of the labeled cDNA from unincorporated $^{32}$P-labeled nucleotides and small cDNA fragments was accomplished by column chromatography. First the beads were separated using the magnetic particle separator and the supernatant (approximate 20 µl) was added to 180 µl of NT2 Buffer. The sample was then placed into a NucleoSpin Extraction Spin Column and centrifuged at 14,000 rpm for 1 min. The content of the collection tube was discarded into the appropriate container for
radioactive waste. The column was washed with 400 µl of NT3 Buffer and centrifuged at 14,000 rpm for 1 minute. The content of the collection tube was discarded into the appropriate container for radioactive waste. The washing with the NT3 Buffer was repeated two more times. The NucleoSpin Column was treated with 100 µl of Buffer NE, allowed to soak for 2 minutes and then centrifuged for 1 minute at 14,000 rpm to elute the purified probe. The radioactivity of the probe was checked by scintillation counting using a 2 µl aliquot in 5 ml of scintillation cocktail.

Hybridization of the cDNA probes to the membrane consisted of several steps. To avoid nonspecific binding, the membrane was prehybridized with hybridization buffer (supplied by CLONTECH) containing sheared salmon testes DNA (0.1mg sheared salmon testes DNA/ml hybridization buffer). This solution was prepared by first heating 2mg (210.52 µl) of sheared salmon testes DNA at 95-100°C for 5 minutes and then chilling it on ice. Next, 20 ml of hybridization buffer (prewarmed at 68°C) was mixed with the sheared salmon testes DNA, to give a final of concentration 0.1 mg/ml. The membrane was then prehybridized for 30 minutes with the salmon sperm DNA solution at 68°C with continuous agitation. Meanwhile 100 µl of cDNA probe was treated with 5 µl of Cₐt-1 DNA, incubated for 2 minutes in boiling water bath, and then incubated on ice for exactly 2 minutes for DNA denaturation. Following the prehybridization period, the probe was added carefully to the prehybridization buffer, making sure that the concentrated solution was not added directly to the membrane. The membrane was hybridized overnight with continuous agitation, and the next day the membrane was washed 4 times with wash solution 1 (2
x SSC-containing 0.3 M NaCl and 0.03M Na$_2$Citrate-2 H$_2$O, 1% SDS) for 30 minutes and once with wash solution 2 (0.1 SSC, 0.5% SDS) for 30 minutes. All washes were done at 68°C. The final wash was for 5 minutes at room temperature with 2 x SSC. The membrane was wrapped in plastic wrap and exposed to X-ray film for various periods of time.
CHAPTER III

RESULTS AND DISCUSSION

Glutathione S-Transferase Assay

The purpose of this set of experiments was to determine if the different treatment conditions previously described produced an increase in activity of GST since it was reported that both chromium (Dubrovskaya et al., 1998) and BPDE (Hayes et al., 1995) induce GST activity. In performing the assay, the most widely used substrate has been 1-chloro-2,4-dinitrobenzene (CDNB) because it has good specificity for all isoenzymes of GST (Simons et al., 1977). When conjugated with glutathione (GSH) it produces S-(2,4-dinitrophenyl) glutathione (Fig. 14), a compound possessing an absorbance spectrum sufficiently different from that of CDNB to allow for a simple spectrophotometric assay at 340 nm (Habig et al., 1981).

\[ \text{Cl} \quad \text{NO}_2 \quad + \quad \text{GSH} \quad \xrightarrow{\text{GST}} \quad \text{SG} \quad \text{NO}_2 \quad \text{+ HCl} \]

Fig. 14. Conjugation of CDNB with GSH.
Experiments were first conducted to validate the assay. Tumor cells (HT1080) were used initially because they grow much faster than normal human fibroblasts (L266C) and allowed faster data collection. Purified glutathione S-transferase (stock solution 0.2 mg/ml) was also used as a control to insure that the assay was working. Initially, HT1080 cells were plated onto 150 mm dishes, were allowed to grow, and after they were nearly confluent, the cells were trypsinized, and a cell suspension was made using 5ml PBS containing 1mg/ml BSA. Sonication of the HT1080 cell suspension was accomplished using 2x30 second bursts with the sonicator (50 Sonic Dismembrator, model F50, Fisher Scientific) set on 10. The sonicator has settings 1 to 10, 1 corresponding to the lowest power and 10 corresponding to the highest power. The GST activity in sonicated cells was then determined and compared to the activity of pure enzyme which had not been sonicated. The results are presented in Table 2. The blank contained 960µl of phosphate buffer and 40 µl of ethanol and the average was substracted from each reading.

<table>
<thead>
<tr>
<th>Setting of Sonicator</th>
<th>Time (sec)</th>
<th>Sample</th>
<th>Units/ml</th>
<th>±S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>GST</td>
<td>0.0692</td>
<td>0.0051</td>
</tr>
<tr>
<td>10</td>
<td>2x30</td>
<td>HT1080 lysate</td>
<td>0.0155</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

We compared the activity of the pure enzyme with the activity of a cell lysate to see if we could measure activity in cells. Comparing the activity of the pure enzyme (stock solution 0.2 mg/ml in phosphate buffer 0.2 M, pH 6.5) with the
activity of the HT1080 lysate, we observed that the activity of the lysate was much lower. One possibility for this result was the fact that the sonication could be destroying the enzyme. Another possibility is that the GST activity is low in this particular cell line. To test if the sonication was destroying the enzyme, we repeated the experiment, sonicating the pure enzyme on setting #10 for 2x30 sec. The results shown in Table 3 clearly indicate that the sonication destroys enzyme activity. There are several possibilities for the lose in activity. Either the power is too great, the exposure time is too long, or both, and therefore, the activity of the enzyme due to denaturation.

Table 3. GST Activity for Pure Enzyme and Pure Enzyme Sonicated at Setting 10 for 2x30 sec.

<table>
<thead>
<tr>
<th>Setting of Sonicator</th>
<th>Time (sec)</th>
<th>Sample</th>
<th>Units/ml</th>
<th>±S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>GST</td>
<td>0.0785</td>
<td>0.0085</td>
</tr>
<tr>
<td>10</td>
<td>2x30</td>
<td>GST</td>
<td>0.0041</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

We next tried to determine the best setting and time for the sonicator so that the enzyme activity would not be affected, but all cell membranes would be lysed. Initially, we used pure enzyme and chose different settings for the sonicator and different sonication times (see Table 4). When we used setting #2 for 2x30 sec we lost the activity of the enzyme indicating that the exposure time was too long. We tried the same setting for 15 and 20 sec and the activity of the enzyme was preserved. When we raised the sonicator setting to 4, we still had good activity for 2x15 sec. We also tried the sonicator set at #6 for 2x15 sec and we were able to measure significant activity. The conclusion from this set of experiments was that the activity of GST is
maintained when the sonicator is set at #4 or #6 and a 15 second burst is used twice (2x15 sec).

Table 4. GST Activity for Pure Enzyme Using Different Settings and Different Sonication Times.

<table>
<thead>
<tr>
<th>Setting of Sonicator</th>
<th>Time (sec)</th>
<th>Sample</th>
<th>Units/ml ±S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>GST</td>
<td>0.0703 ±0.0020</td>
</tr>
<tr>
<td>2</td>
<td>2x30</td>
<td>GST</td>
<td>0.0110 ±0.0015</td>
</tr>
<tr>
<td>2</td>
<td>2x20</td>
<td>GST</td>
<td>0.0653 ±0.0015</td>
</tr>
<tr>
<td>2</td>
<td>2x15</td>
<td>GST</td>
<td>0.0806 ±0.0037</td>
</tr>
<tr>
<td>2</td>
<td>2x10</td>
<td>GST</td>
<td>0.0756 ±0.0054</td>
</tr>
<tr>
<td>4</td>
<td>2x25</td>
<td>GST</td>
<td>0.0607 ±0.0008</td>
</tr>
<tr>
<td>4</td>
<td>2x15</td>
<td>GST</td>
<td>0.0901 ±0.0039</td>
</tr>
<tr>
<td>6</td>
<td>2x15</td>
<td>GST</td>
<td>0.0618 ±0.0021</td>
</tr>
</tbody>
</table>

Next, we needed to check these settings and times using cell lysates. From one 150 mm dish of nearly confluent HT1080 cells we made a cell suspension as previously described. Before sonication, we divided the sample into three equal aliquotes. GST was determined for each in triplicate, using the sonicator set at #4 and a pulse time of 15 sec (Table 5, Set 1). Lysates were prepared using 2 other 150 mm dishes, and analyzed in the same manner (Table 5, Set 2 and Set 3). We found that the activity of GST was consistent for each set of cell samples showing that the assay gave reproducible results for a single sample. The values of activity differ for different sets of data because the number of cells per culture dish varies. An additional dish of cells was used to prepare lysates using setting #6 pulsed 2x15 sec. The result of the GST assay gave an activity of 0.1660 units/ml. Thus, increasing the setting did not increase
the activity. We concluded from this set of experiments that maximal cell lysis was probably accomplished using setting 4 and the activity of the enzyme was preserved.

Table 5. GST Activity for HT1080 Cell Lysate Using Setting #4, 2x15 sec.

<table>
<thead>
<tr>
<th>Setting of Sonicator</th>
<th>Time (sec)</th>
<th>Sample</th>
<th>Units/ml</th>
<th>±S.D.</th>
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</thead>
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<tr>
<td></td>
<td>4</td>
<td>2x15 HT1080</td>
<td>0.1525</td>
<td>0.0100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>lysate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Set 1</td>
<td></td>
<td>4</td>
<td>0.1723</td>
<td>0.0048</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2x15 HT1080</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>4</td>
<td>0.1776</td>
<td>0.0041</td>
</tr>
<tr>
<td></td>
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<td>2x15 HT1080</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>lysate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Set 2</td>
<td></td>
<td>4</td>
<td>0.0679</td>
<td>0.0055</td>
</tr>
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<td>2x15 HT1080</td>
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<td></td>
<td>lysate</td>
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<td>4</td>
<td>0.0802</td>
<td>0.0085</td>
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<td>2x15 HT1080</td>
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<td>4</td>
<td>0.0717</td>
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<td>2x15 HT1080</td>
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<td></td>
<td></td>
<td>lysate</td>
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<td></td>
</tr>
<tr>
<td>Set 3</td>
<td></td>
<td>4</td>
<td>0.1331</td>
<td>0.0005</td>
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<td>2x15 HT1080</td>
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<td>2x15 HT1080</td>
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<td></td>
<td></td>
<td>lysate</td>
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</tr>
</tbody>
</table>

We also performed some experiments to verify that the compounds we were testing did not interfere with the assay. We first tested the absorption of chromium at the wavelength used in the assay to check if at 320 nm Cr (VI) does exhibit absorption. The control contains 760 µl phosphate buffer (pH 6.5), 40 µl of CDNB, 100 µl of GSH and 100 µl of PBS. Chromium, at a final concentration of 0.3 µM, was added to the same mixture as the control and the assay was performed in triplicate for
each condition. The results are presented in Table 6 and show that chromium does not give significant absorbance under the conditions of our assay.

Table 6. Absorption of Cr at 340 nm.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Units/ml</th>
<th>±S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
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<td>0.0005</td>
</tr>
<tr>
<td>Chromium</td>
<td>0.0047</td>
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</tbody>
</table>

We next determined if the carcinogens we used affected the assay. This time we used the normal human fibroblasts cell line L266C. Cells were grown, harvested while in logarithmic growth, and lysed by the sonication procedure chosen above. The lysate was used for determination of the activity of the enzyme in the presence and absence of Cr and BPDE. The carcinogens were added to the lysate at a final concentration of 0.3 µM (the same as we used when we treated cells with carcinogens) and then the assay was performed. The results from two sets of experiments are presented in Fig. 15 with error bars representing the range of the data obtained. The activity of each sample is relative to the untreated control lysate which have been defined as 100%. These experiments show that the glutathione S-transferase assay is not affected by Cr and/or BPDE at the concentrations we used in the experiments to follow. Therefore, residual Cr and BPDE in the cell lysate should not influence the GST assay.
Fig. 15. Effect of Added Cr and BPDE on GST Activity.

Having established the assay, we performed experiments using normal human fibroblasts (L266C cells). We set up the four conditions described in the Introduction (control, chromium treated cells, BPDE treated cells, and cells treated with both carcinogens). Cells in the logarithmic phase were trypsinized, washed off the dishes
with PBS (with 1mg/ml BSA) and lysed by sonication on setting #4 for 2x15 sec. Three sets of experiments were performed and the results are presented in Fig. 16 with error bars representing the standard deviation. The conclusion from these experiments is that glutathione S-transferase activity is not influenced by any of our conditions.

Fig. 16. GST Activity in Various Sonicated Cell Lysates.

We also performed the same experiment as in Figure 16 except using the freeze/thawing method for preparing cell lysates. The cells were plated in 3 150 mm
dishes per condition, and when they reached about 50% confluence, they were treated using one of the four conditions previously described. Cells were harvested, and lysed by three cycles freeze/thawing using a dry ice/ethanol bath. The experiment was performed three times and the results are presented in Fig. 17 with error bars representing the standard deviation. This set of experiments shows once again that the

Fig. 17. GST Activity in Various Cell Lysates Prepared by the Freeze/thawing Method.
activity of glutathione S-transferase is not influenced by the different conditions of treatment used in this study.

Some research groups have observed an increase of expression of hGSTP1-1 in cells treated with BPDE (Fields et al., 1998). For this particular study human T47D cells (breast) were stably transfected with hGSTP-1. These investigators used a higher concentration of BPDE 1.0 µM and a different exposure time (20 min) (Fields et al., 1998) and found that expression of hGSTP1-1 protects against DNA damage induced by BPDE. Since we could not express the GST activity as a function of protein in the sample we cannot compare (numerically) our results with theirs. In addition, the cell line that was used by Fields et al., T47D, was different from those used in this study. Therefore, we don’t know if GST can be induced in fibroblasts.

It has also been reported that Cr (VI) induces GST activity (Tully et al., 2000). These workers used HepG2 cells and only found induction (6 fold) using 10 µM Cr (VI) for 48 hours, but not at concentrations of 5 µM or less. For this particular study the exposure time was the same as the one we used, but they used a hepatoma cell line. They did not measure GST activity but gene expression. Thus, comparisons of activities cannot be made.

There are groups who have measured the GST activity in the same organ type and the values reported are very different, even though they used the same method to assay the activity of the enzyme. For example, Singh’s group (Singh et al., 1998), determined the GST activity in mice liver to be 470 nmol/min/mg protein while Kim’s lab (Kim et al., 1997), reported that the GST activity was 60 nmol/min/mg
protein using the same tissue. These values correspond to the control cells in their studies, so it would seem reasonable to compare. Therefore, the determined activities reported in the literature vary considerably and one cannot speculate about the ability of glutathione S-transferase to be induced in fibroblasts by chromium or BPDE based upon literature values.

Cell Cycle Analysis

One hypothesis, that could explain the previous mutant frequency results discussed in the Introduction, is that the cell cycle is halted at the G1/S boundary in the presence of chromium and/or the BPDE and chromium so that DNA repair can occur prior to DNA replication. To investigate this possibility, the incorporation of tritiated thymidine into DNA was measured. Cells were treated as described in Materials and Methods so that the four conditions were established (control, chromium treated cells, BPDE treated cells, and chromium and BPDE treated cells). Cells were assayed for $^{3}$H-thymidine incorporation at 12, 16, 20, 24, 28, 32, 36 and 40 hours after release from confluence and the average and standard deviation for triplicate samples were plotted. The results show that entry into S phase occurs after about 30 hours for control cells (Fig. 18), cells treated with chromium (Fig. 19), cells treated with BPDE (Fig. 20) and cells treated with both carcinogens (Fig. 21). This data suggests that the cell cycle doesn't seem to be affected by the different conditions since the entry into the S phase occurs at the same time for all conditions.
Delay of S phase may not be the cause of the antagonistic effect of chromium on relative mutant frequency of BPDE.

Fig. 18. Entry into S Phase for Control Cells
Fig. 19. Entry into S Phase for Cr Treated Cells.
Fig. 20. Entry into S Phase for BPDE Treated Cells.
Fig. 21. Entry into S Phase for Cells Treated with Both Carcinogens.

The results of this experiment are consistent with previous data from laboratory (see Fig. 6). In previous experiments, control cells exhibited the highest level of cloning and here, the control cells show the highest incorporation of $^3$H-thymidine. Chromium treated and BPDE treated showed a reduction in cloning
ability, and the reduction is mirrored in the incorporation of $^3$H-thymidine (Table 7). The greatest effect on cloning is observed when cells are treated with both carcinogens and we see the lowest incorporation under these conditions.

Table 7. Cell Cycle Analysis for Different Conditions.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Maximal Incorporation (CPM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>$6684.57 \pm 2592.65$</td>
</tr>
<tr>
<td>Cr</td>
<td>$1937.50 \pm 406.55$</td>
</tr>
<tr>
<td>BPDE</td>
<td>$729.40 \pm 130.10$</td>
</tr>
<tr>
<td>Cr+BPDE</td>
<td>$309.12 \pm 15.57$</td>
</tr>
</tbody>
</table>

Xu et al., performed studies to see if there is a delay in cell cycle when cells are treated with Cr(VI). They used LL 24 cells, which are a fibroblast-like normal human lung cell line. Treatment conditions were 0, 10, 50 and 200 µM Cr(VI) for 2 hours. Cells were harvested at 6, 24 and 48 hours after treatment. The samples were analyzed on an EPICS Profile Flow Cytometer. They observed a slightly reduced progression into S phase. We can say, however, that their results were obtained at much higher chromium concentrations than we used in our study. Again it is difficult to compare their results with ours since they used different concentrations, different exposure times, and a different assay.

Binkova’s laboratory focused on the effect of benzo(a)pyrene on human embryonic lung diploid fibroblasts (HEL cells) (Binkova et al., 2000). The exposure time was 24 hours using B(a)P at 1.0 µM. Data acquisition was performed using an
argon laser fluorescence activated cell analyser. After 24 hours of treatment, there was an increase in the proportion of cells which had entered the S phase compared to controls. For the control they observed 88% of the cell population in the G₀/G₁ phase and 8% in S phase. The cells treated with B(a)P (0.10 µM), 84.5% cells were in the G₀/G₁ phase and 12% were in S phase. Again, it is difficult to compare situations when you use different cell lines, different concentrations, and exposure times. However, one should note that the effects of cell cycle arrest were observed at BPDE concentrations higher than those used in our study.

In the future, to see if our results are reproducible, the experiment can be repeated using flow cytometry for data acquisition. Using the DNA labeling assay we could follow only the transition to S phase, but by flow cytometry, you can get information about the cell populations in various stages of the cell cycle.

**p53 and p21 Protein Level**

As discussed in the Introduction, chemical damage to DNA may not result in a mutation if the DNA is repaired prior to replication (Bertram, 2001). There are checkpoints in the cell cycle at the G₁/S boundary to make sure that the DNA is integer, and if not, the entry into the S phase may be delayed to allow more time for DNA repair. It has been shown that an important role in halting the cell cycle at the G₁/S phase is played by p53, which induces the synthesis of p21, which inhibits the formation of the Cyclin D: CDK4/CDK6 complex (Xu et al., 1996).
Western blotting analysis was used to determine if p53 and p21 are induced under various conditions in our system. Four conditions were established. Cells were treated with either normal growth media for 48 hours (control), media containing 0.3 µM potassium dichromate for 48 hours, normal growth media for 46 hours followed by media containing 0.3 µM BPDE for 2 hours (BPDE), or 46 hours with media containing 0.3 µM potassium dichromate followed by 2 hours with media containing both 0.3 µM chromium and 0.3 µM BPDE together. Cell lysates were prepared as described in Materials and Methods, and the amount of total protein was determined for each lysate to ensure that the same amount of protein was analyzed in each condition. Western Blot analysis for p53 and p21 was performed as described in Materials and Methods using 10 µg total lysate in each well of the gel. The amount of protein loaded into the wells of the gel was low compared to other groups (Binkova et al., 2000). Unfortunately we could not load more into the wells.

The results (Fig. 22), showed that the assay was working since the positive control was visible, but no bands were seen in our samples. Only the positive control for p53 protein was ever observed in any experiment. Molecular weight markers were included on the gel to help us to identify the bands (not shown).

Unfortunately, when we probed for p21 nothing was detected. We could not determine if the assay was working since we did not have an available positive control. The reason that we did not observe any bands for p53 and p21 may be that the level of protein expression was below the detectability of the assay. In the future, a more concentrated lysate can be prepared or use immunoprecipitation can be used.
By precipitating the proteins of interest (p53 or p21) with antibodies directed against them prior to running the gel. We did not attempt this because we moved on to the microarray assay which contained both p21 and p53.

Fig. 22. Western Blot Analysis of p53 Expression. Lane 1 was loaded with the positive control for p53 and the remaining samples were loaded as follows: lane #2 - control lysate, lane #3-Cr treated cell lysate, lane #4-BPDE treated cell lysate and lane #5-both carcinogens treated cell lysate.

As with GST induction, we were not able to duplicate results previously reported by others. Binkova and collaborators using human embryonic lung diploid fibroblasts (HEL) observed an induction of p53 after treatment with 1.0 µM B(a)P for
24 h (Binkova et al., 2000). Again, the conditions used by these investigators resulted in a much greater exposure than what we have used in our studies.

**General Gene Expression**

When proteins are expressed at low levels, an alternative is to look at the gene expression. The purpose of the following experiments was to determine if there are genes that are induced or suppressed by our different conditions. For these experiments, we used a commercially available kit (Atlas Select Human Tumor Array from Clontech CA), which contains 437 differentially expressed cDNAs including p53 and p21. The array also contains 61 novel sequences, 32 cDNAs from genes with established importance in cancer, 16 having partial homology to known sequences, 9 having homology to known ESTs (Expressed Sequence Tags), and 365 other known genes. There are also housekeeping genes like ubiquitin, phospholipase A2, α-tubulin, β-actin and glucose-3-phosphate dehydrogenase (G3PDH) spotted on the membrane.

One set of cells was treated for 46 hours with media containing potassium dichromate followed by 2 hours with media containing both chromium and BPDE and another set of cells was treated with normal growth media for 48 hours (control). The RNA was isolated from the cells as described in Materials and Methods. The A\textsubscript{260/280} ratio was 2.00 for the control cell RNA and 2.03 for the RNA from cells treated with both carcinogens. The quality of the RNA was checked by running a gel and the samples were compared with previous samples prepared using a different method.
protocol for RNA isolation (GIBCO BRL-Life Technologies- TRizol reagent). Ten µg of RNA was loaded in each well. It was necessary to isolate the RNA using the CLONTECH protocol because other commercially available kits for RNA isolation are not compatible with this array, resulting in a high level of nonspecific background. The gel is presented in Fig. 23 and shows that the quality of RNA isolated is good.

Fig. 23. RNA Purity Following Isolation (1). The lanes were loaded as follows: control (1) and both carcinogen treatment (2) from using TRizol reagent and control (3) and both carcinogen treatment (4) from samples using the CLONTECH protocol.
Fig. 24. RNA Purity Following DNase Treatment. The lanes were loaded as follows: control (1) and both carcinogen treatment (2) from using TRIzol reagent and control (3) and both carcinogen treatment (4) from samples using the CLONTECH protocol after the DNase treatment.

Next, the samples were treated with DNase as described by the manufacturer and the quality of the RNA checked. The $A_{260/280}$ ratio was 2.04 for the control cell RNA and 2.02 for the RNA from cells treated with both carcinogens. A picture of this gel is presented in Fig. 24. As we can see, a smear appears in the DNase treated samples instead of the bands meaning that the RNA was degraded during the DNase treatment.
To solve this problem, we incubated the RNA samples with DNase 1 and ANTI-RNase (Ambion, TX), at a concentration 1 unit/µl. Fig. 25 shows the samples before DNase treatment. Five µg of RNA were loaded in each well. The $A_{260/280}$ ratio was 2.17 for the control cell RNA and 1.95 for the Cr+BPDE cell RNA.

![RNA purity following isolation](image)

Fig. 25. RNA Purity Following Isolation (2). The first lane is the control RNA sample (1) and the second lane is RNA isolated from both carcinogen treatment (2).

The RNA following the DNase treatment using Ambion’s ANTI-RNase is presented in Fig 26. Five µg of RNA were loaded in each well. The $A_{260/280}$ ratio was 2.37 for the control RNA and 2.22 for Cr+BPDE treated cell RNA. We can see from
Fig. 26 that the quality of RNA after DNase treatment is significantly better when the DNase treatment is done in the presence of Ambion ANTI-RNase.

![Image of gel electrophoresis](image)

**Fig. 26.** RNA Purity Following DNase Treatment in the Presence of Ambion ANTI-RNase. The first lane is a positive control from an old sample (1), the second lane is the control RNA sample after DNase treatment in the presence of Ambion ANTI-RNase. (2) and the third lane is RNA isolated from both carcinogen treatment after DNase treatment in the presence of Ambion ANTI-RNase (3).

Once the RNA degradation problem was solved we proceeded to make probes from our RNA. To verify the probe labeling procedure, we synthesized cDNA from our two samples as described in Materials and Methods, as well as, from a control RNA provided by the manufacturer which contains a 1:1:1 mixture of human, mouse
and rat total RNA. An aliquot of 2 µl of each sample was diluted in 5 ml of scintillation liquid and counted. The counts for the three samples are presented in Table 8. The results, shown in Table 8, indicate significant incorporation of label into all three samples.

Table 8. Labeling of cDNA Probe.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Counts/min (CPM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control RNA/Company</td>
<td>40302.0</td>
</tr>
<tr>
<td>Control Sample RNA</td>
<td>46591.0</td>
</tr>
<tr>
<td>Cr+BPDE Sample RNA</td>
<td>28838.0</td>
</tr>
</tbody>
</table>

It was also necessary to verify that we could hybridize the cDNA probes onto the membranes. The company provided Control PolyA⁺ RNA. After making labeled probe from this RNA, the probe was hybridized to the membrane. The amount of radiation incorporated into the probe for the control PolyA⁺ RNA was 354368.0 counts/min (CPM). Notice that the amount of radiation incorporated is about 10 times more than that of our samples, but the protocol predicted better incorporation for the control polyA⁺. The probe was then hybridized to the Atlas membrane as described in Materials and Methods. Following hybridization, the membrane was washed and exposed to film for three days (see Fig. 27). We were not able to see the housekeeping genes which help to orient the film on the grid to identify other genes of interest as in Fig. 28. (G1-G21). Fig. 29 shows the results of a membrane exposed for three days using an Atlas Human cDNA expression array and Human Control
polyA+ +. The darker dots on the bottom and right side are the housekeeping genes, which help to orient the film onto the grid. Notice that for this example there were double spots for each gene, but for the tumor array we used there should be single spots.

We did attempt to hybridize the probes made from our samples to the membranes. The signals for the control cells and cells treated with both carcinogens were too weak, and only four dots were visible after 3 days of exposure. Thus, we have not able to identify differences between the two conditions tested using this assay.

Using the Atlas Select Tumor Array we tried to identify genes that are turned on/off by our different treatments. Unfortunately, the hybridization of the cDNA probe to the membrane was not good and we could not observe a signal. This may have been because we did not use enough hybridization buffer. We used only 5 ml as described in the instructions, but used a larger hybridization bottle. Another reason may be that the membranes were used several times and they lost their quality. We were also not sure about the quality of the cDNA probe synthesized. When we check it by running an agarose gel, we observed most of the label at the bottom of the gel, indicating very small pieces. Maybe this problem can be overcome if we try to reduce the RNA degradation by adding 100 units of Ambion’s ANTI-RNase after adding the magnetic beads to the RNA sample.
Fig. 27. Analysis of Control RNA.
Fig. 28. Grid for the CLONTECH Atlas Select Human Tumor Array.

Fig. 29. Example of Results Obtainable Using Atlas Select Array System.
CHAPTER IV

CONCLUSION

The purpose of this study was to determine what protective mechanisms may be triggered in cells in response to multiple carcinogen treatment. First we tested if the enzyme glutathione S-transferase, which is involved in detoxification of a wide variety of chemicals, is induced by our treatments. We prepared cell lysates using two methods (sonication and freeze/thawing method), but we could not observe any significant increases in GST activity for any of our treatments.

We also tested if the different treatments with carcinogens had an effect on the cell cycle. Using tritiated thymidine we performed DNA labeling experiments for the four conditions and observed that cells enter S phase at about the same time, regardless of the treatment.

We were not able to come to any conclusions from the gene array experiments since our hybridizations did not give strong enough signals.
Statistical analysis was done for the GST experiments using the two-way RGLM ANOVA. For all the experiments tested, the p value was larger than .05. Thus, the conclusion is that the results for different treatments have no statistically significance difference in induction of GST compared to the control.


conjugation of (+)-Anti-7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo(a) pyrene. *Archives of Biochemistry and Biophysics*, **353**, 337-348.