The Effect of Diethylstilbestrol on p53 Expression in Liver and Neural Tissue of the Female Hamster (*Mesocricetus auratus*).

Jeffrey L. McPherson

ABSTRACT

Diethylstilbestrol (DES) is carcinogenic and a known endocrine disruptor. Studies have demonstrated that perinatal exposure to DES results in fertility deficits and abnormalities of the reproductive tract. P53 is a known tumor suppressor. It is a tumor suppressor in that it halts abnormal cell growth and initiates programmed cell death if DNA damage has occurred. Studies have shown that after DNA damage, levels of p53 and its transcriptional activity rise dramatically. This research focused on the disruptive activity of DES and sought to find a correlation between perinatal DES injections and increased p53 expression in liver and neural tissues, which would be an indicator that DES was causing DNA damage in these tissues. This study was performed in the golden syrian hamster due to its ease of use. An experimental group was given DES injections perinatally, and a control group was given corn oil injections. At maturity, the liver and neural tissues were surgically harvested. The tissues were separated into four fractions; cytosolic, microsomal, nuclear extract, and nuclear pellet. Proteins were separated using electrophoresis and analyzed using western blotting. No evidence of p53 was found in the liver and neural tissues.

Keywords: *DES, diethylstilbestrol, cancer, p53, tumor suppressor genes*

INTRODUCTION

Diethylstilbestrol (DES) is a synthetic estrogen known to be a carcinogen in humans and rodents (McLachlan, 1976). In the mid 1900's, DES was often prescribed for the support of high-risk pregnancies (Herbst et al, 1979). The occurrence of a rare cancer in women exposed to DES in utero was found in 1971 by Herbst et al (Herbst et al, 1971). Clinical and experimental animal studies have since demonstrated that perinatal exposure to DES results in fertility deficits and abnormalities of the male and female reproductive tract (Marselos, 1992). Thus, DES is now classified as an endocrine disruptor (Colburn et al, 1993). It is very beneficial to examine the impact of DES on tissues throughout the body, as this allows us to be able to determine if the disruption caused by perinatal DES exposure is specific to reproductive tissues, or if the DES has effects on other tissues during development as well.

P53 is a known tumor suppressor. The proliferation of normal cells is thought to be regulated by growth-promoting oncogenes counterbalanced by growth-constraining tumor suppression genes. Mutations create oncogenes that force the growth of tumor cells. Conversely, genetic disruptions that inactivate tumor suppression genes liberate the cell from the constraints imposed by these genes, yielding the unconstrained growth of the cancer cell. Initial studies seemed to demonstrate p53 as an oncogene, but this was inaccurate. Initial studies were performed using p53 cDNA clones that were actually a mutant strain, and wild-type cDNAs were later found to be strongly growth suppressive showing p53 to be a tumor suppressor. It has been shown to play a role in transcription, in cell cycle control, and in many other metabolic functions. It is a tumor suppressor in that it halts abnormal growth in normal cells and thus prevents cancer. However, a small change in p53, a mutation of one of its 393 amino acids, can eliminate the protein's capabilities and allow a cancer to grow (Koshland, 1993).

About 50 percent of all human cancers contain a mutation in p53, so one hopes that the molecule will provide new insight into treating the disease (Koshland, 1993). 51 different types of cancers have been found to carry p53 mutations, including brain and liver cancer (Culotta and Koshland, 1993).

Work suggests that the normal p53 protein serves to protect the genome against DNA damaging events. It apparently has the capacity to sense when damage occurs and then halt cell division until the damage is repaired, or, if the damage is too severe to be corrected, initiates programmed cell death. When the gene is mutated, the cells may keep dividing, allowing DNA damage to build up, and the mutated p53 may even spur additional abnormal growth (Marx, 1993). Researchers have also found that the p53 protein's tumor suppression effects may depend on its activity as a transcription factor, the first step in gene activity.

In 1993, several groups of scientists showed that after DNA damage, levels of p53 protein and its transciptional activity rise dramatically. This research focuses on the disruptive activity of DES and seeks to find a corrlelation between perinatal DES injections, and increased p53 expression. This would indicate that DES is causing DNA damage within normal cells.

Figure 1: Surgical removal of tissues a)Exposure of the brain b)Exposure of the liver

Hendry (1999)has shown DES to be disruptive in uterine tissues, and the focus of this research is to determine if that is an isolated effect, or if DES is causing disruption throughout other tissues of the hamster.

MATERIALS AND METHODS

Golden Syrian hamsters were divided into four groups, two experimental groups and two control groups.   The experimental groups were given perinatal DES injections and the control groups were given corn oil injections.   Both groups were then ovarectomized and allowed to grow to maturity.   One set of experimental and control hamsters were allowed to reach six months of age and the other two months of age.   Both sets had reached sexual maturity at this time. The older group is used to see if more dramatic effects occur due to DES over extended time periods.
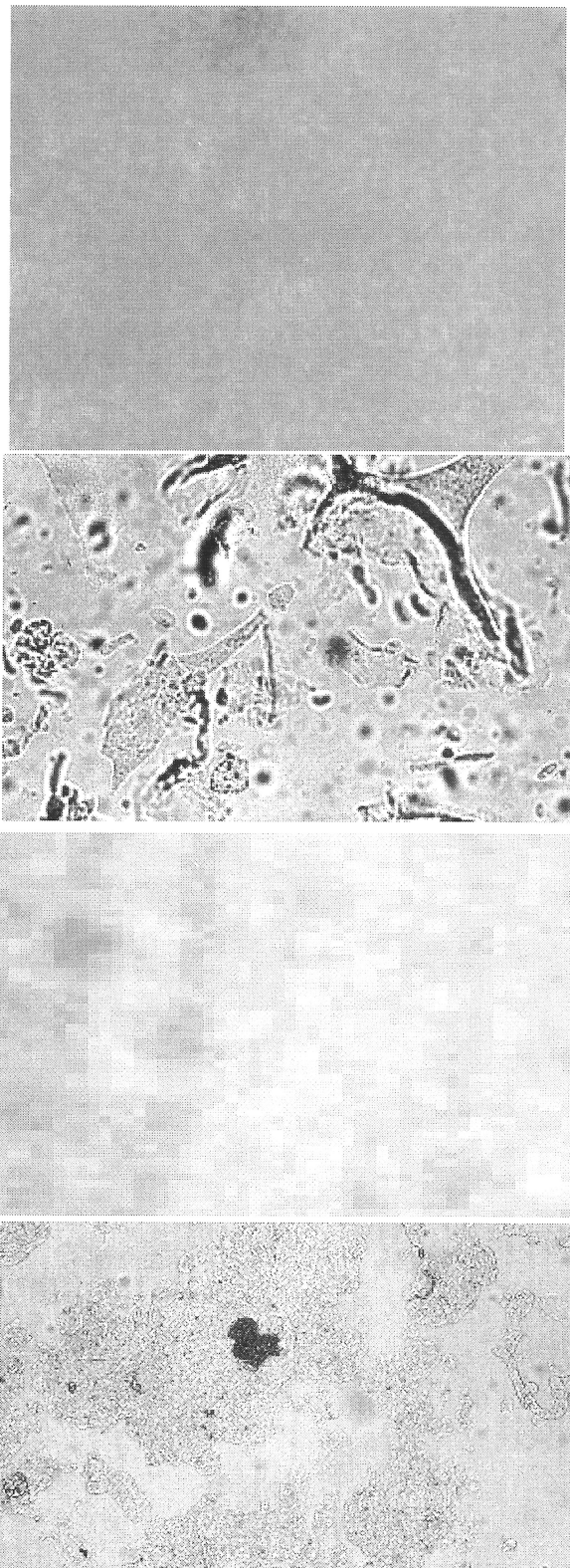

Figure 2: Photomicrographs of cellular fractions a)cytosolic 400X b)microsomal 400X c)nuclear extract 400X d)nuclear pellet 200X

Brain and liver tissues were then harvested from each of the subjects (Fig 1). The animals were anesthetized and euthanized, and the tissues were taken surgically . The liver tissues were sliced into thin pieces and incubated on ice in at least three volumes saline to allow blood to drain from the tissues. The brain tissue was incubated on ice in the saline intact. The incubation process was repeated using three changes of saline and the tissues were then weighed out, and placed into a TE buffer solution(10 mmol Tris-HCl, 1mmol EDTA). The amount of buffer used was determined according to the weight of each tissue sample (buffer volume equal to nine times tissue weight, e.g. 1 gram tissue,
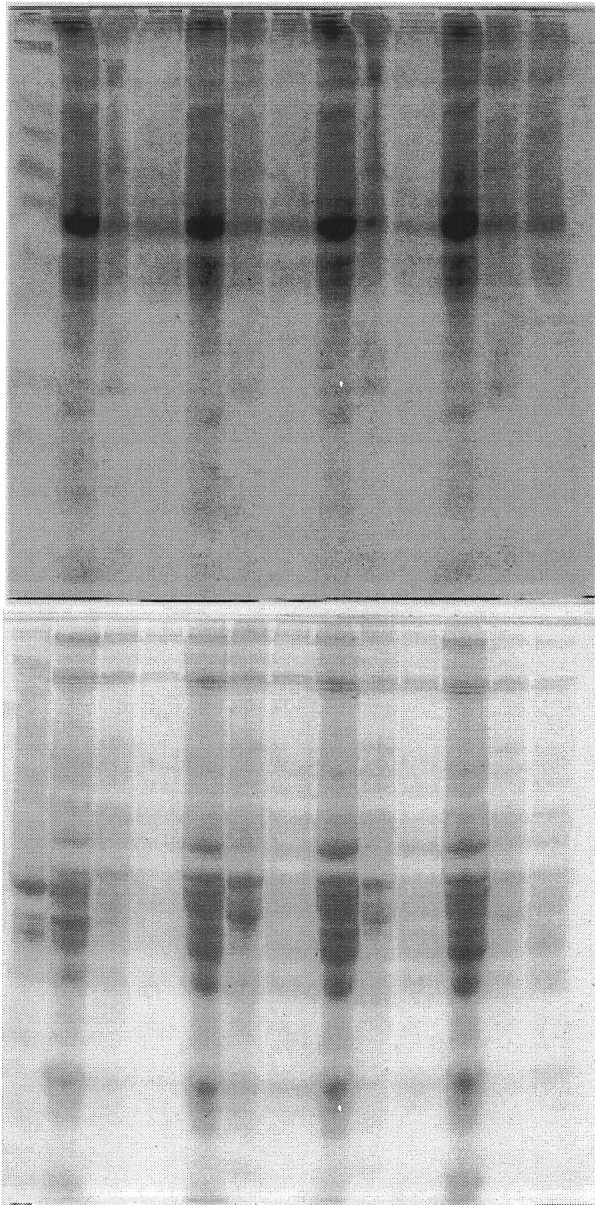
9 mL TE).

The samples were homogenized using a hand-held homogenizer, and placed in centrifuge tubes. The samples were spun at 2500 rpm for 10 minutes in a SS34 rotor. The supernatant from this spin contains the cytosol and microsomal components. The pellet contains nuclear material.

The supernatant from the first spin was then poured off, brought to its designated volume with TE, and spun again at 15000 rpm for 15 minutes. This separated the cytosolic and microsomal components. These components were then put into separate tubes and their volume was once again brought to their designated level.

The pellet containing nuclear material from the initial spin was resuspended in a TE/KCL buffer(10 mmol Tris-HCl, 1 mmol EDTA, 300 mmol KCl) and allowed to incubate for 15 minutes. This allowed for the nuclear proteins to be released from the nuclear membrane. This solution was spun at 15000 rpm for 15 minutes. This step sufficiently separated the solution into a nuclear extract component and a nuclear pellet. Both components were then brought to their designated volume with TE.

Each of the four fractions (cytosol, microsomal, nuclear extract, nuclear pellet) were then aliquoted for later use and frozen (Fig 2).

The next step was to perform polyacrylamide gel electrophoresis on the protein samples. The protein samples are prepared by adding 4X sample buffer (0.25 M Tris, pH 6.8, 40% glycerol, 20% beta-mercaptoethanol, 4% sodium dodecyl sulfate) to the protein solutions at a concentration of 1volume buffer: 3 volumes protein solution. The samples are then boiled to denature the proteins and the samples are loaded into the gel wells (50 microliters) and run for 18 hours at 60 volts. The fractions were run against a marker with proteins of known molecular weight in order to be able to determine the range in which p53 should be located. Only the cytosolic, microsomal, and nuclear extract fractions were used in this study because p53 has not been shown to be present in the nuclear pellet.

After electrophoresis, the final step is to perform a western blot. The polyacrylamide gel is incubated for 1 hour in electrotransfer buffer (192 mM glycine, 25 mM Tris base, 15% methanol, 0.02% SDS). A nitrocellulose membrane and filter paper cut to the size of the gel are also saturated in the buffer. The transfer of the proteins from the gel to the millipore nitrocellulose membrane is accomplished using an owl separation system run at 400 mA for 1.5 hours. The nitrocellulose membrane is then rinsed in five washes of TTBS buffer (100 mM Tris base, 0.9% NaCl, 0.1% Tween 20) and blocking is performed in in TTBS with 5% non-fat dry milk for 0.5 hours. The membrane is then rinsed again in TTBS five times. The next step is to incubate the membrane for 4 hours with the primary antibody specific for p53. The antibody used is p53 (Ab-3) monoclonal mouse IgG
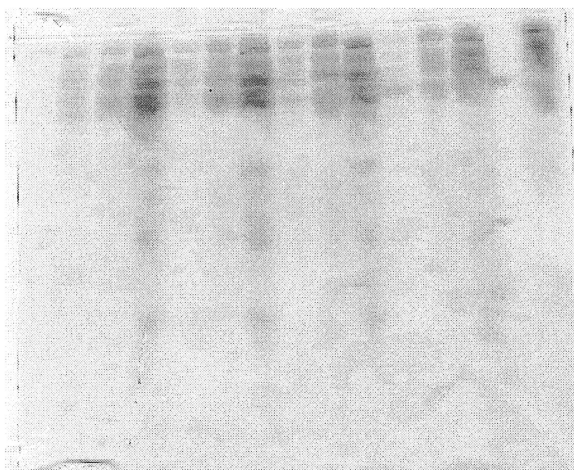

Figure 3: Polyacrylamide gels demonstrating protein separation. Lane 1-marker; 2,5,8,11-cytosol; 3,6,9,12-microsomal; 4,7,10,13-nuclear extract; 2-7 DES; 8-13 Control. Top)brain Bottom)liver

Figure 4: Example of polyacrylamide gel stained after transfer of proteins (liver).

manufactured by Oncogene Research Products. The working solution consisted of 150 microliters of antibody solution (100 microgram/ml) in 15 ml TTBS. After incubation with the primary antibody, the membrane is again rinsed five times in TTBS, and incubated with a secondary antibody for 2 hours. The secondary antibody is specific for mouse IgG, the primary antibody. The secondary antibody used was whole molecule anti-mouse IgG manufactured by Sigma Chemical Co. The secondary antibody must be incubated with ExtrAvidin alkaline phosphatase conjugate (Sigma Chemical Co.) for 0.5 hours prior to use. The working solution of secondary antibody consisted of 12 microliters anti-mouse IgG (1.27 mg/ml) and 6 microliters ExtrAvidin in 24 ml of TTBS. The membrane is then rinsed five times in TTBS. Finally, color develpoment is accomplished using Sigma Fast 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium. One tablet dissolved in 10 ml deionized water yields the desired working concentration (0.15 mg/ml BCIP, 0.30 mg/ml NBT, 100 mM Tris buffer, 5 mM $MgCl_2$). The membrane was incubated in this solution until color development took place. The appearance of bands are used for analysis.

A control blot was also performed by following the above procedure, with the omission of the primary antibody step. The secondary antibody is aimed towards mouse IgG, but cross reactions can occur with hamster IgG, which would be in any blood in the tissues. A comparison of the control blot to the sample blots will allow for determination of banding due to p53, and those due to cross reactions of the secondary antibody.

RESULTS

Each of the tissues were successfully extracted, compartmentalized into cellular fractions, and separated by electrophoresis. Evidence of protein separation is clear in the coomassie stained polyacrylamide gels for each tissue indicated by the unique banding patterns of each fraction (Fig 3).

The proteins were then successfully transferred to the nitro cellulose membrane evidenced by the reduction of protein concentration in a gel stained after blot transfer (Fig 4), and by the appearance of protein bands on the membrane after color development of the western blot.
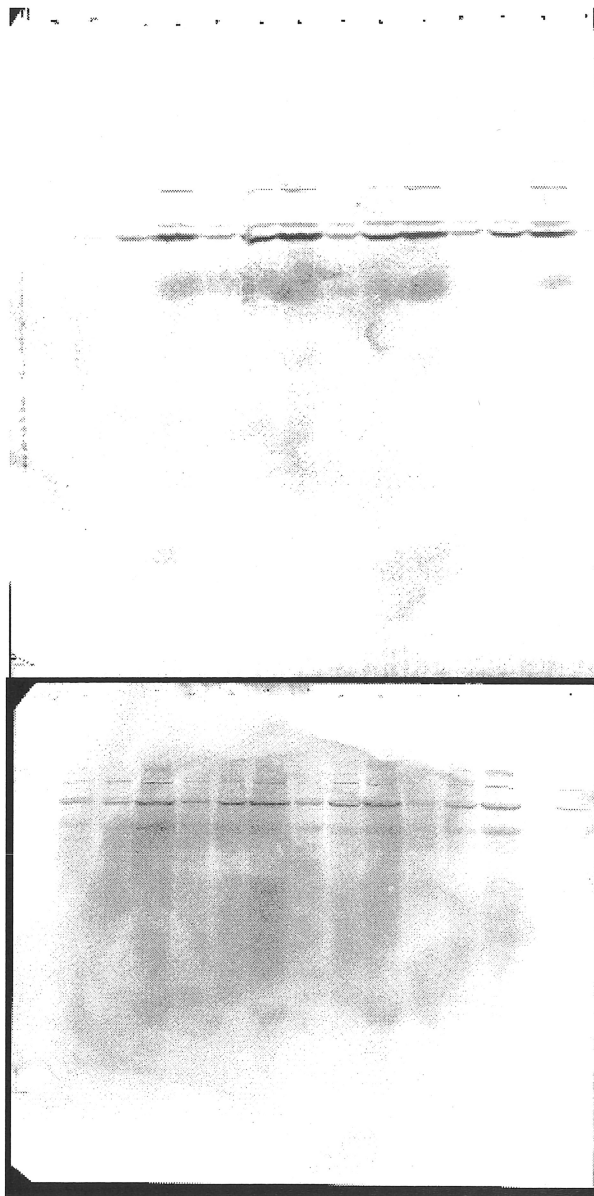

Figure 5: Western blots. Lane 2,5,8,11-nuclear extract; 3,6,9,12-microsomal; 4,7,10,13- cytosol; 15-marker. Top)brain Bottom)liver
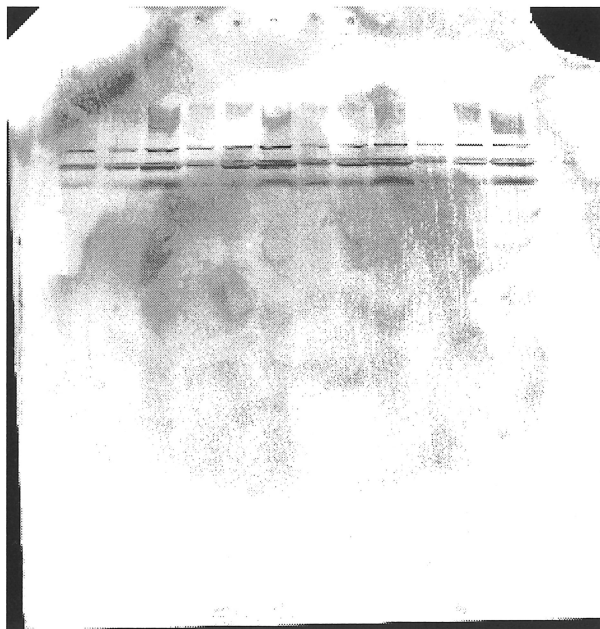
Figure 6: Control blot - no primary antibody used.

A comparison between the western blots performed on the liver proteins, brain proteins, and the control blot showed that all of the bands on the liver and brain blots were due to cross reactions between the secondary anti-mouse IgG and hamster IgG. By comparing the location of the bands on the western blot to a marker transferred to the same membrane, it was also shown that each of the visible bands was due to a protein of molecular weight too low to be p53. No additional bands were visible to support evidence for the presence of p53 (Fig 5).

DISCUSSION

The lack of an evident indication of the presence of p53 poses some interesting questions. There is a possibility that the concentration of p53 in the brain and liver tissues is so low that it was not picked up by my analytical method. It could also be that DES caused no DNA damage in these tissues and p53 expression was in no way stimulated by the injections, therefore normal p53 levels were retained.

There is also a possibility that my procedural methods caused a misrepresentation as to the amount of p53 present. It is possible that the p53 was retained in the nuclear pellet, as it is a nuclear protein, and not analyzed.

ACKNOWLEDGEMENTS

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