Mechanism of Genotoxicity of Diethylstilbestrol in Vivo*

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Diethylstilbestrol (DES) is a carcinogen in humans and rodents which has eluded mechanistic clarification of its carcinogenic action. In vitro and in vivo, binding of DES to DNA has been found previously, but covalent DNA adducts could not be identified. In this study, the nature of binding was investigated by 32P-postlabeling, a rapid and highly sensitive assay for covalent DNA damage, to distinguish between a genotoxic or epigenetic mechanism of carcinogenesis by DES. A unique and distinct DNA adduct pattern was observed in kidney, liver, uterus (or testes) of female (or male, respectively) Syrian hamsters treated with a single injection of DES (200 mg/kg body weight). This set of DNA adducts closely matched patterns generated in vitro by reaction of diethylstilbestrol-4',4"-quinone with DNA or 2',3'-deoxyguanosine 3'-monophosphate. The major and several minor DES-DNA adducts in vivo had identical chromatographic mobilities in 11 different solvent systems with corresponding adducts obtained in vitro. The major adduct spot, generated in vitro by reaction of diethylstilbestrol-4',4"-quinone and DNA, was chemically unstable (half-life at 37 °C: 4–5 days). The persistence in vivo of these DNA modifications was low (biological half-life: 14 h) presumably because of chemical instability in concert with DNA repair. After injection of identical dosages of DES, adduct concentrations were 4–6-fold higher in females than in males. These results demonstrate that DES is capable of covalently modifying DNA. Moreover, diethylstilbestrol-4',4"-quinone is the major reactive metabolic intermediate responsible for the genotoxic activity of DES. Tumors are expected to arise only in rapidly dividing cells due to the short biological lifetimes of DES-DNA adducts.

Diethylstilbestrol (DES) is a known carcinogen in humans and in rodents (1). The biochemical mechanism of DES-induced carcinogenesis has been considered to be epigenetic in nature because of the high hormonal potency of this synthetic estrogen and because covalent DES-DNA interaction could not be detected despite intensive searches in several laboratories (2). Radioactively labeled DES was found to bind to DNA in vitro (3–5) and in vivo (6), but the nature of binding could not be clarified in any of these studies. The difficulties experienced in defining the nature of DES-DNA interaction were partially explained by the instability of DNA binding of DES Q, a metabolic intermediate of DES, in vitro (7) and of DES in rats and hamsters in vivo (6). Noncovalent intercalation was also investigated but was not observed for DES (8) or its more planar metabolic intermediate DES Q (7).

In contrast, a number of biological and biochemical effects of DES were noted which depended on metabolic activation of the stilbene and are commonly associated with genotoxic activity (reviewed by Metzler (9)). These include: induction of sister chromatid exchange (10, 11), of unscheduled DNA synthesis (12), and of aneuploidy in Syrian hamster embryo cells (13). These DES-induced genetic alterations, usually indicative of a genotoxic agent, as well as the covalent interaction of the metabolic intermediate DES Q with peptides and proteins (7) suggested a renewed study of the nature of binding of DES to DNA by 32P-postlabeling analysis, a more rapid and more sensitive assay procedure (14, 15) than those used previously (3–5). These examinations were carried out with DNA isolated from DES-treated Syrian hamsters, a species prone to DES-induced cancer (16). Covalent DES-DNA adducts were detected in hamster liver, kidney, and uterus after a single injection of DES. These adducts were identified by cochromatography with adducts obtained by in vitro reactions of DES Q and DNA from untreated hamsters.

**MATERIALS AND METHODS**

**Chemicals**—The major deoxyribonucleoside 3'-monophosphates and DES were purchased from Sigma. DES Q, diethylstilbestrol-3,4-epoxide, and Z,Z-dienestrol were prepared as described previously (17, 18). 3'-Hydroxydiethylstilbestrol was a gift of Dr. Pat Murphy, Eli Lilly and Company, Indianapolis, IN. DNA was isolated from hamster organs by a modification of Marmur's procedure (19). Highly purified hamster liver DNA was found to carry less contamination and background radioactivity in 32P-postlabeling analysis than commercial nucleic acids. Carrier-free [32P]phosphate, 300 mCi/ml in water, was purchased from ICN Biochemicals, Irvine, CA. Material and chemicals needed for the 32P-postlabeling assays were obtained from sources described previously (14, 15, 20, 21).

**DES Treatment of Animals**—Three-month-old male and female adult Syrian hamsters weighing approximately 160 g (purchased from Harlan/Sprague Dawley, Houston, TX) or 10-day-old neonates received intraperitoneal injections of DES (200 mg/kg body weight dissolved in corn oil). Four h post injection, the animals were killed by decapitation. Livers, kidneys, uteri (or testes, respectively) were removed quickly, frozen in liquid nitrogen, and stored in a −75 °C freezer. Tissues from age-matched untreated animals served as controls.

**Binding of Stilbenes to DNA in Vitro**—Hamster liver DNA (2.5 mg/ml of distilled water) was sheared by five passages through a 25-gauge needle and then denatured by incubation at 100 °C for 15 min. The mixture was cooled immediately in an ice bath. The reaction mixture containing 1 mg of DNA, 1.6 mg of DES Q, 3'-hydroxydiethylstilbestrol, or diethylstilbestrol-3,4-epoxide, respectively, in 1 ml of water:acetone:chloroform (2:2:1) was incubated at 37 °C for 45 or 120 min. Control reactions were carried out with DES or Z,Z-dienestrol under the same conditions. Reaction mixtures were extracted with diethyl ether (10 x). The DNA was precipitated with ethanol at
**TABLE I**

Chromatographic conditions for $^{32}$P analysis of DES-DNA adducts

<table>
<thead>
<tr>
<th>Solvent number</th>
<th>Solvent composition</th>
<th>Stationary phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1 M sodium phosphate, pH 6.8</td>
<td>PEI*</td>
</tr>
<tr>
<td>II</td>
<td>3.8 M lithium formate/6.8 M urea, pH 3.5</td>
<td>PEI</td>
</tr>
<tr>
<td>III</td>
<td>0.7 M sodium phosphate/7 M urea, pH 6.8</td>
<td>PEI</td>
</tr>
<tr>
<td>IV</td>
<td>1.7 M sodium phosphate, pH 6.0</td>
<td>PEI</td>
</tr>
<tr>
<td>V</td>
<td>2-Propanol/4 M ammonia (2.8:2.2, v:v)</td>
<td>PEI</td>
</tr>
<tr>
<td>VI</td>
<td>2-Propanol/4 M ammonia (3.1:1.5, v:v)</td>
<td>PEI</td>
</tr>
<tr>
<td>VII</td>
<td>Ethanol/3 M ammonium formate, pH 3.6</td>
<td>PEI</td>
</tr>
<tr>
<td>VIII</td>
<td>0.4 M ammonium formate, pH 6.2</td>
<td>C$_{18}$</td>
</tr>
<tr>
<td>IX</td>
<td>2-Propanol/0.4 M ammonium formate, pH 6.2 (1:1, v:v)</td>
<td>C$_{18}$</td>
</tr>
<tr>
<td>X</td>
<td>2-Propanol/0.2 M ammonium formate, pH 6.2 (1.5:5.5, v:v)</td>
<td>C$_{18}$</td>
</tr>
<tr>
<td>XI</td>
<td>2-Propanol/4 M ammonia (11:9, v:v)</td>
<td>Silica</td>
</tr>
</tbody>
</table>

* PEI, polyethyleneimine.

**RESULTS**

DES-DNA Adducts in Vivo and in Vitro—Because of previous failures to detect DES-DNA adducts (3-6), large dosages of DES were injected in hamsters. Compared to controls which showed only background radioactivity, liver, kidney, and uterine DNA from DES-treated hamsters contained additional adduct spots when analyzed by $^{32}$P postlabeling (Fig. 1). The highest concentration of DES-DNA covalent modifications was in liver (1 adduct/0.9 x 10$^8$ nucleotides), slightly less in kidney (1 adduct/1.6 x 10$^8$ nucleotides) (Table II). In uterine DNA, only the major adduct spot 4 could be detected at low concentrations (1 adduct/7.8 x 10$^8$ nucleotides) (Fig. 1 and Table II).

Adducts obtained in vitro by reaction of DNA with 3'-hydroxydiethylstilbestrol or diethylstilbestrol-3,4-epoxide, previously postulated putative reactive intermediates of DES (23, 24), differed from in vivo adduct patterns with respect to number of modified nucleotides and chromatographic mobilities (Fig. 2). Control DNA did not show any DNA adducts under these conditions (data not shown). The latter in vitro
Modified nucleotides (Fig. 2, A and C) were not investigated further, since they did not match those observed in vivo. Adduct patterns in liver and kidney of DES-treated hamsters closely matched those obtained by reaction of DES Q and DNA in vitro (Fig. 2B). They were similar with respect to spot intensities and chromatographic mobilities for the major and several minor adducts. Kidney DNA contained one additional modified nucleotide (spot K1) (Fig. 1E). In hepatic DNA there were only minor amounts of radioactivity in addition to that resembling the pattern of DES Q. In control reactions with DES Q and Z,Z-dienestrol (data not shown), only background radioactivity could be detected.

The adduct-forming potential of DES Q was examined further with the four major deoxyribonucleoside 3'-monophosphates. 32P-Postlabeling analysis of the products of the reaction of DES Q and dGMP (Fig. 3B) gave one major (spot 4) and three minor (spots 1, 2, and 3) adducts, which closely matched the pattern seen for spots 1, 2, and 3, respectively. These data demonstrated that DES Q is a genotoxic metabolite of DES and that it possesses high reactivity with guanine nucleotides. Higher concentrations of adducts are achieved in reactions with DNA than with mononucleotides, presumably because steric interactions with DNA facilitate covalent binding.

Identification of DES-DNA Adducts—The similarities of adduct patterns in DNA of DES-treated hamsters (Fig. 1, D and E) with those obtained in vitro by reactions of DES Q with DNA (Fig. 2B) or dGMP (Fig. 3B) prompted additional experiments aimed at identifications of adducts. The major DES-DNA adduct spot 4 obtained in vivo (Fig. 1) and the major DES Q-DNA adduct spot 4 obtained in vitro (Fig. 2) were examined by cochromatography experiments and compared to each other using a total of 11 different chromatographic systems (listed in Table I) on polyethyleneimine-cellulose, C18 reversed phase, or silica gel thin-layer plates (Figs. 4 and 5). In the first set of experiments, adduct spot 4, eluted from 32P-postlabeling maps of liver DNA of DES-treated hamsters (shown in Fig. 1D), and the major adduct obtained from in vitro reactions of DES Q and DNA (shown in Fig. 2B) were cochromatographed in several solvent systems (Fig. 4). The adducts were also compared to other modified nucleotides observed in this area of polyethyleneimine-cellulose maps such as KI (Fig. 1E) and KII (see below). In none of these chromatographic systems was there a difference in chromatographic mobilities between DES Q-DNA nuc...

Fig. 2. 32P-Postlabeling analysis of DNA covalently modified by incubation with 3'-hydroxydiethylstilbestrol (A), DES Q (B), and diethylstibestrol-3,4-epoxide (C). Purified hamster liver DNA (1 mg) was incubated with 1.6 mg of stilbene reactive intermediate for 45 min at 37 °C. The DNA was then isolated and analyzed by the nuclease P1 procedure (20) of the 32P-postlabeling assay as described previously (14, 15). Autoradiography was carried out with Cronex film at -75 °C for 1 h. The major (spot 4) and minor adducts formed by DES Q are labeled to the right of each spot.

Fig. 3. 32P-Postlabeling analysis of the products of the reaction of dGMP and DES (A) or DES Q (B) and of 2'-deoxyadenosine 3'-monophosphate and DES Q (C). 2'-Deoxyribonucleoside 3'-monophosphate and DES or DES Q were incubated in water/acetone/chloroform for 2 h. The products of the reactions were analyzed by the nuclease P1 procedure (20) of the 32P-postlabeling assay as described previously (14, 15). Chromatography was on polyethyleneimine-cellulose with solvents I and III (see Table I) from bottom to top and from left to right, respectively. Autoradiography was carried out with Cronex film using Du Pont Lighting Plus intensifying screens (-75 °C, 1-h exposure time). In B, adducts are lettered to the right of each spot.
adduct spot 4 obtained in vitro and the major DES-DNA adduct generated in vivo. This identity with respect to chromatographic mobility and peak shape is illustrated for solvent systems II, III, and VI. In a second set of experiments, the identities of the major spot 4 and minor DES-DNA adducts observed in DNA of DES-treated hamsters were examined and compared to adducts of DES Q obtained in vitro (Fig. 5). The chromatographic mobilities were examined on silica gel and polyethyleneimine-cellulose sheets using solvents III, VI, and XI. In all three experiments, the corresponding DNA adducts were found to have identical chromatographic mobility. When DNA samples from in vitro and in vivo experiments were mixed and analyzed, adduct mobilities and peak shapes were indistinguishable from those obtained in separate experiments. Identical mobilities were also observed in other chromatographic systems on polyethyleneimine-cellulose or C18 reversed phase plates (solvents V, and VII–X, data not shown). These results demonstrate that DES Q is the major reactive adduct-forming intermediate observed in DNA of DES-treated hamsters. These adduct patterns are unique and cannot be generated by other reactive DES metabolites (Fig. 2).

Stability and Persistence of DNA Adducts—When reaction times of the incubations of DES Q and DNA were varied with the aim of optimizing adduct yields, it was noted that adduct patterns changed. Optimal adduct concentrations were achieved by incubating at 37 °C for 24 h. After a prolonged reaction time, intensities of the major adduct spot 4 and most minor spots were lower (data not shown). Moreover, additional spots were formed. Concentrations of these additional spots peaked and also decreased after extended incubation times (data not shown). These analyses demonstrate that DES Q-DNA adducts are chemically unstable and decompose with time and/or that adducts rearrange into isomeric adduct spots which are also unstable and spontaneously decompose. The instability of DNA adducts was further investigated by isolating adducted DNA after reaction with DES Q for 24 h and performing DNA adduct analysis after incubation in a water-acetone solvent mixture for various lengths of time at 37 °C. The half-life of the major adduct (spot 4) was determined to be between 4 and 5 days (data not shown).

The persistence of DES-DNA adducts in vivo was investigated to assess the biological significance of DES-DNA adduct formation. Concentrations of adduct spot 4, the only covalent modification detected in testes of male Syrian hamsters, decreased by 50% over a period of 14 h which was more rapid than expected from rates of chemical decomposition observed at 37 °C in vitro. The radioactivity content of spot 4 decreased from 121 cpm 2 h post injection to 62 cpm 16 h post injection. No adducts could be detected after 1 and 3 weeks post injection (data not shown). These data demonstrate that chemical instability of adducts and DNA repair in vivo are synergistic in rapidly decreasing DES-DNA adduct levels. Comparable decreases in DNA adduct levels as a function of time were observed in other hamster organs (data not shown).

Sex Differences in Adduct Levels—Sex differences in adduct levels were also investigated. In kidney and liver DNA of male and female hamster neonates at 10 days of age, which have received injections of identical dosages of DES, spot 4 was the only adduct observed except for an additional spot KII in female kidney (data not shown) which was not identical with spot KI in adult kidney (Fig. 1E). There were profound differences in concentrations of spot 4 between males and females. Adduct levels were 6-fold higher in liver (521 cpm) and 4-fold higher in kidney (402 cpm) of females than in the corresponding organs of males (83 and 103 cpm, respectively). These data demonstrate that female hamsters are much more susceptible to DES genotoxicity than males.

DISCUSSION

The lack of success with attempts to demonstrate the covalent nature of DES binding to DNA (3–6) led to the classification of DES as an epigenetic carcinogen (2). In particular, it was thought that hormonal imbalance or an uncontrolled hormonal stimulation of cell proliferation was the dominant aspect of carcinogenesis by this stilbene estro-
patterns induced by a variety of estrogens, including DES, specifically in the hamster kidney as published previously (28-30). The DES-DNA adducts described in this report could not be detected in previous ³²P-postlabeling maps of kidney DNA from hamsters treated chronically with DES for 5 months (28-30). Reasons for this difference presumably include (i) the lower dosage of estrogen when DES is administered chronically by a subcutaneous pellet (28-30) resulting in an absorption of approximately 60μg/animal/day (31), (ii) changes in renal estrogen metabolism resulting in decreased DES Q formation at this 5-month time point (32-34), and (iii) the low persistence of DES-DNA modifications resulting in low steady state concentrations of DNA adducts.

The low persistence of DES-DNA adducts is likely caused by the chemical instability of these compounds, demonstrated in vitro, and probably is exacerbated by DNA repair in vivo. The resulting short biological half-life of DNA damage may have been one of the reasons for the lack of success in demonstrating covalent DNA addition compounds although binding of DES (3-6) or DES Q (8) to DNA could be detected.

The biological role of DES-DNA adduction still remains to be clarified. The DES-DNA adducts shown in this report were generated by high doses of DES which may have altered the pharmacokinetics of this stilbene and the spectrum of its metabolites produced. Therefore, an etiological role of DNA modification in DES-induced tumors still needs to be demonstrated particularly at the lower dosages commonly used in tumor induction experiments. Nevertheless, the characteristics of DES-DNA adducts in hamsters shown in this report correlate well with two unusual features of DES-associated cancer in humans. 1) The adduct instability in vitro and low persistence in vivo may explain that tumors did not arise in DES-treated women, but in their daughters (35, 36), presumably because unstable DNA damage produced long-lasting biological effects only in rapidly dividing cells (of the fetus). In hamsters, DES-DNA adducts were observed in all organs examined as shown here. If they also form in all organs in humans, they may decompose in maternal tissue before cell division incorporates erroneous messages. In contrast, in rapidly dividing fetal cells, DNA adduct formation may be considered to be a tumor-initiating event. Thus, DES-DNA adduction is postulated to be a necessary but not sufficient event for the development of cancer by DES. 2) The higher adduct levels in females compared to those in males correlate well with the higher incidence of DES-associated cancer in women. In human males, genital tract abnormalities were observed, levels in females compared to those in males correlate well and probably is exacerbated by DNA repair in vivo.

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In summary, the data presented here support a mechanism of genotoxicity of DES via initial metabolic oxidation of this stilbene estrogen to DES Q. This reactive metabolic intermediate may then covalently bind to DNA. Covalent DNA modification has been shown to occur in many cells and tissues, but likely decreases rapidly with time because of repair and concurrent chemical instability of adducts. Tumors, if they arise from this DNA modification, are expected to be formed only in rapidly dividing cells due to the short biological lifetimes of DES-DNA adducts. The hormonal potency of this synthetic estrogen may in addition effect transformation of initiated cells and subsequent growth of hormone-responsive tumors.

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REFERENCES