Biochemical Studies of Diethylstilbestrol-induced Kidney Tumors in the Golden Syrian Hamster

Teresa Lacomba and Maria Gabaldón
Servicio de Cancerología Experimental, Facultad de Medicina, Paseo al Mar, Valencia, Spain

SUMMARY

Hepatic glucuronyltransferase activity toward diethylstilbestrol (DES) decreased in hamsters treated chronically with DES (3.5 and 7 months). The presence of both primitive estrogen-dependent renal tumors and transplanted estrogen-independent tumors resulted in an increase of glucuronyltransferase activity toward DES. This activity was higher in the hamster kidney than in the rat kidney. Chronic administration of DES dimethyl ether produced renal tumors in the male hamster similar to those produced by DES, although a longer period of treatment was required. DES monoglucuronide administered for 15 months did not produce renal tumors. Hepatic β-glucuronidase activity towards DES monoglucuronide was not induced in hamsters treated with DES monoglucuronide. Hepatic O-demethylase activity toward DES dimethyl ether was not induced in hamsters treated with DES dimethyl ether. This activity was not modified by the presence of a renal tumor.

INTRODUCTION

Chronic administration of DES induces renal tumors in the male hamster and in the ovariectomized female hamster. This phenomenon has not been described in other animals and is of great interest as it is an estrogen-induced tumor in an organ which is not normally regarded as belonging to the endocrine system. The characteristics of the tumor and the influence of different variables on its formation have been reviewed by Kirkman (12). Horning (9) formulated the hypothesis that the hamster liver has a smaller DES conjugation capacity than do the livers of other rodents. This fact would cause nonconjugated high DES levels to be eliminated, thereby becoming an irritative stimulus to the hamster kidney, which would eventually lead to tumor formation.

To confirm this hypothesis, we studied the influence of sex, species, castration in males, and DES pellet implantation upon the formation of DESGA by hamster liver homogenates (5). Our results did not agree with Horning's hypothesis, as we found glucuronyltransferase activity toward DES in the hamster to be from 5 to 6 times higher than in the rat, and, in the hamster, castration in males and sex did not result in statistically significant differences in DESGA formation.

The major question focuses on the relationship, if any, between the levels of glucuronyltransferase activity toward DES and the induction of renal tumors; for this reason, we have studied the enzyme levels in hamster and rat kidneys and the effect of chronic administration of DES (3.5 and 7 months) on the hepatic glucuronyltransferase in the hamster. Since the induction of renal tumors in the hamster by DES derivatives has not been studied, we have considered it worthwhile to study the induction of renal tumors in the hamster by 2 DES derivatives, a physiological one, DESGA, and a nonphysiological one, DESDME.

Glucuronides are generally devoid of pharmacological action. However, O-glucuronides of N-hydroxy metabolites of carcinogenic aromatic amines possess unexpected chemical reactivity with tissue nucleophiles, and they are now considered ultimate carcinogenic metabolites of aromatic amines (11). We have investigated the tumorigenic activity of DESGA on the hamster kidney, taking into account the fact that DESGA is the main metabolite of DES, that its elimination is performed through the urine, and that its formation by hamster liver is much greater than by rat liver.

DES, DESGA, and DESDME have been administered chronically to male hamsters in order to compare their tumorigenic activity on the kidney. At the end of the treatment, the activities of the enzymes that act on the products administered were determined in the liver in order to ascertain whether the observed effects should be attributed to the original product or to its metabolites if the formation of these was intense.

Activity of UDP glucuronate glucuronyltransferase (acceptor unspecific), EC 2.4.1.17, toward DES in hamsters treated with DES, of β-glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.31) toward DESGA in hamsters treated with DESGA, and of O-demethylase toward DESDME in hamsters treated with DESDME have been determined in liver homogenates. The determination of these 3 activities in nontreated hamsters gives the induction capacity of DES, DESGA, and DESDME on the enzymes that act on them.

MATERIALS AND METHODS

Chemicals. DES, UDPGA, glucose 6-phosphate, NADP*, glucose 6-phosphate dehydrogenase (Type XV), and Triton X-100 were provided by Sigma Chemical Company (St. Louis,
Mo.). DESDME was provided by Nutritional Biochemicals Corporation (Cleveland, Ohio), and 25-mg DES pellets (Cyren A) were provided by Bayer (Leverkusen, Germany). DESGA was obtained from the urine of rabbits fed DES at a daily rate of 500 mg for 5 days following the procedures given by Dodgson et al. (2).

Animals. Male Wistar rats and golden Syrian hamsters were used in this study. For the induction of renal tumors in the hamster, 0.2 ml of a 11.2 mM suspension of DES, DESDME, and DESGA in the vehicle was administered s.c. 3 times a week (every other day). The vehicle was 1% carboxymethylcellulose N-1000 in 0.9% NaCl solution. The administration of DES, DESDME, and the vehicle was started on 15-day-old hamsters and, in the case of DESGA, on 30-day-old hamsters. From the 6th month of treatment, groups of hamsters were sacrificed every month in order to investigate the presence of renal tumors. The transplants were performed s.c. from an estrogen-independent tumor obtained in our laboratory by serial transfers through hamsters of a primitive renal tumor produced by chronic administration of DES.

Assay of Glucuronidase Activity. The technique described by Gabaldón et al. (6) was used with the following modifications. The reaction was stopped by addition of 5 ml of 20% trichloroacetic acid:ethanol (1:4), and the precipitate was separated by filtration 15 min later. The incubation mixture was composed of Tris, 35 mM; UDPGA, 0.42 mM; MgCl₂, 10.5 mM; DES, 0.149 mM; propylene glycol, 3% v/v; 15% homogenate, 1 ml (final volume, 5 ml; pH, 7.3). DES was added to the incubation mixture dissolved in 60% propylene glycol. DESGA identification was accomplished by the procedure described previously (5).

Assay of β-Glucuronidase Activity. Hamsters were sacrificed by cervical dislocation, and the livers were immediately removed and washed with 0.15 M KCl:0.32 mM KHCO₃ solution at 0°C. Then, 1% homogenates were prepared with water at 0°C. The incubation mixture was composed of acetate buffer, 70 mM; DESGA, 0.4 mM; ethanol, 3% v/v; 1% homogenate, 1 ml (final volume, 5 ml; pH, 4.5). DESGA was added to the incubation mixture dissolved in 30% ethanol. In the assays performed with Triton X-100, the final concentration in the incubation mixture was 0.1% v/v. The mixture was incubated with shaking at 37°C for 90 min. The reaction was stopped by addition of 5 ml of 20% trichloroacetic acid:ethanol (1:4), and the precipitate was separated by filtration 15 min later; 5 ml of freshly prepared 2% NaHCO₃ were added to an aliquot of 5 ml from the filtrate. The mixture was extracted twice with 10-ml portions of diethyl ether. The ether was removed on the water bath, and the aqueous ethanolic residue was evaporated in a vacuum at a temperature below 45°C; 1 ml of 30% ethanol, 4 ml of 4% Na₂CO₃·10H₂O in N NaOH, and 0.25 ml of N Folin-Ciocalteu’s reagent were added to the solid residue. The contents were shaken, and readings were taken at 540 μm after 30 to 45 min at 20°C. A DES standard curve was prepared as described in Ref. 6. The amount of DES formed was calculated by difference with a blank incubated without substrate and with DESGA added after protein precipitation. Assays were done in triplicate.

RESULTS

Production of Renal Tumors. Between the 7th and 8th month of DES treatment, renal tumors began to appear, and by the 10th month all the hamsters had developed tumors. After the 15th month of DESGA treatment, none of the hamsters had developed tumors, nor had any of those treated with the vehicle alone. Between the 8th and 9th month of DESDME treatment, renal tumors began to appear, and by the 12th month 80% of the remaining hamsters had developed tumors (Table 1). The renal tumors produced by DESDME are smaller than those produced by DES. The histological characteristics of both tumors are similar.

Glucuronyltransferase Activity toward DES. In the presence of UDPGA, glucuronyltransferase activity in kidney homogenates was approximately 5 times higher in the hamster than in the rat (Table 2). The relation of the renal activities in these 2 animals is similar to that observed in the liver (5). In the absence of added UDPGA (endogenous activity), the differences of activity were not statistically significant.

In both the presence and the absence of UDPGA, DES treatment reduced glucuronyltransferase activity. This effect became more obvious as the treatment progressed (Table 3). The hamsters with estrogen-dependent renal tumors had the same hepatic glucuronyltransferase activity as the controls. However, taking into account the fact that DES treatment decreased the activity and that hamsters treated 7 months with DES but without having developed renal tumors had an activity from 2 to 3 times lower than the controls, we
Estrogen-induced Kidney Tumors in Hamsters

Table 1

Occurrence of renal tumors in hamsters treated with DES, DESDME, and DESGA

<table>
<thead>
<tr>
<th>Mo. of treatment</th>
<th>DES (54)</th>
<th>DESDME (59)</th>
<th>DESGA (43)</th>
<th>Vehicle (62)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6—7</td>
<td>0/5 (0%)</td>
<td>0/5 (0%)</td>
<td>0/5 (0%)</td>
<td>0/5 (0%)</td>
</tr>
<tr>
<td>7—8</td>
<td>8/13 (62%)</td>
<td>0/4 (0%)</td>
<td>0/5 (0%)</td>
<td>0/10 (0%)</td>
</tr>
<tr>
<td>8—9</td>
<td>22/25 (88%)</td>
<td>1/3 (33%)</td>
<td>0/5 (0%)</td>
<td>0/10 (0%)</td>
</tr>
<tr>
<td>9—10</td>
<td>11/11 (100%)</td>
<td>3/8 (37%)</td>
<td>0/4 (0%)</td>
<td>0/10 (0%)</td>
</tr>
<tr>
<td>10—11</td>
<td>13/29 (45%)</td>
<td>0/12 (0%)</td>
<td>0/10 (0%)</td>
<td>0/10 (0%)</td>
</tr>
<tr>
<td>11—12</td>
<td>8/10 (80%)</td>
<td>0/12 (0%)</td>
<td>0/17 (0%)</td>
<td>0/17 (0%)</td>
</tr>
</tbody>
</table>

a A 11.2 mM suspension (0.2 ml) of the products in the vehicle was administered 3 times a week (every other day).
b Numbers in parentheses, number of animals treated with each product.

Table 2

Glucuronyltransferase activity in kidney homogenates

<table>
<thead>
<tr>
<th>Animals</th>
<th>No. of animals</th>
<th>Without UDPGA</th>
<th>p&lt;sup&gt;b&lt;/sup&gt;</th>
<th>With UDPGA</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamster</td>
<td>8</td>
<td>25.7 ± 21.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>N.S.</td>
<td>87.5 ± 17.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Rat</td>
<td>8</td>
<td>11.0 ± 14.7</td>
<td>N.S.</td>
<td>18.0 ± 17.2</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

a Five-month-old rats and hamsters.
b Level of significance, Student's t test; p > 0.005 is considered not significant (N.S.).
c Values are given in micromoles of DES consumed per 100 mg of fresh liver per 90 min.
Results are given as the mean ± S.D.

Table 3

Glucuronyltransferase activity in hamster liver treated with DES

<table>
<thead>
<tr>
<th>Animals</th>
<th>No. of animals</th>
<th>Mo. of treatment</th>
<th>Without UDPGA</th>
<th>p&lt;sup&gt;a&lt;/sup&gt;</th>
<th>With UDPGA</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>3.5</td>
<td>65.5 ± 12.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;0.001</td>
<td>201.5 ± 15.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treated</td>
<td>10</td>
<td>3.5</td>
<td>35.5 ± 15.0</td>
<td></td>
<td>128.2 ± 24.7</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>45.0 ± 18.2</td>
<td></td>
<td>&lt;0.005</td>
<td>151.5 ± 17.5</td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>9</td>
<td>7</td>
<td>18.7 ± 9.5</td>
<td>&lt;0.001</td>
<td>68.0 ± 13.0</td>
<td></td>
</tr>
</tbody>
</table>

a Level of significance, Student's t test.
b Four-month-old hamsters receiving the vehicle alone.
c Values are given in micromoles of DES consumed per 100 mg of fresh liver per 90 min.
Results are given as the mean ± S.D.
d Four-month-old hamsters receiving 0.2 ml of a 11.2 mM suspension of DES in the vehicle 3 times a week (every other day).
e Eight-month-old hamsters receiving 25-mg DES pellets at 22 days, 4 months, and 7 months. None of the hamsters developed renal tumors.

conclude that the presence of the tumor increases activity. The presence of estrogen-independent transplanted tumor increased the activity, and this increase was of the same order as that observed in the estrogen-dependent renal tumor (Table 4).

DES consumption differences in the absence and the presence of UDPGA were statistically significant in the hamster in all the cases studied and not significant in the rat.

β-Glucuronidase Activity toward DESGA. There are no references concerning the existence in the hamster of the β-glucuronidase endogenous inhibitor described in other species (19). The first assays of activity were performed in the presence and in the absence of Triton X-100 in order to ascertain its effect on the possible endogenous inhibitor. The presence of Triton X-100 had not caused differences in the enzyme activity with homogenates either at 1 or 5%; however, the dilution of the homogenate resulted in a considerable activity increase. Hepatic β-glucuronidase activity toward DESGA was not induced by DESGA treatment lasting 10 months (Table 5).

O-Demethylase Activity toward DESDME. Hepatic O-demethylase activity was not induced by DESDME.
Table 4

<table>
<thead>
<tr>
<th>Animals</th>
<th>No. of animals</th>
<th>Type of tumor</th>
<th>Without UDPGA</th>
<th>p</th>
<th>With UDPGA</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td></td>
<td>45.0 ± 18.2b</td>
<td>N. S.</td>
<td>151.5 ± 17.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>With transplanted tumorc</td>
<td>10</td>
<td>Estrogen-independent</td>
<td>28.7 ± 12.7</td>
<td></td>
<td>222.2 ± 37.7</td>
<td></td>
</tr>
<tr>
<td>Controld</td>
<td>11</td>
<td></td>
<td>73.0 ± 28.7</td>
<td>N. S.</td>
<td>209.5 ± 43.7</td>
<td></td>
</tr>
<tr>
<td>With renal tumore</td>
<td>11</td>
<td>Estrogen-dependent</td>
<td>60.2 ± 22.2</td>
<td></td>
<td>209.2 ± 54.0</td>
<td></td>
</tr>
</tbody>
</table>

a Level of significance, Student’s t test; p > 0.005 is considered not significant (N.S.).
b Values are given in mmoles of DES consumed per 100 mg of fresh liver per 90 min. Results are given as the mean ± S.D.
c Eight-month-old hamsters. The determinations were performed 36 days after the s.c. transplant of an estrogen-independent tumor. The weight of the tumor was 7.13 ± 2.25 g.
d Nine-month-old hamsters receiving the vehicle alone.
e Nine-month-old hamsters receiving 0.2 ml of an 11.2 mM suspension of DES in the vehicle 3 times a week (every other day).

Table 5

<table>
<thead>
<tr>
<th>Animals</th>
<th>No. of animals</th>
<th>β-Glucuronidasea</th>
<th>p</th>
<th>O-Demethylasec</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>1585.2 ± 175.3</td>
<td>N. S.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated with DESGAe</td>
<td>11</td>
<td>1723.3 ± 182.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>44.0 ± 7.1</td>
<td>N. S.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated with DESDMEf</td>
<td>10</td>
<td>52.4 ± 8.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Activity is given in mmoles of DES formed per 100 mg of fresh liver per 90 min. Results are given as the mean ± S.D.
b Level of significance, Student’s t test; p > 0.005 is considered not significant (N.S.).
c Activity is given in mmoles of formaldehyde formed per 100 mg of fresh liver per 90 min.
d Eleven-month-old hamsters treated 10 months with DESGA. The dose was 0.2 ml of an 11.2 mM suspension of DESGA in the vehicle 3 times a week (every other day).
e Eleven-month-old hamsters treated for 10.5 months with DESGA. The dose was 0.2 ml of an 11.2 mM suspension of DESDME in the vehicle 3 times a week (every other day). Six hamsters developed renal tumors. The presence of renal tumors did not cause statistically significant differences in the formation of formaldehyde.

DISCUSSION

Estrogens in general decrease glucuronyltransferase activity, but this effect seems to depend on the duration of treatment. In the hamster, the implantation of a DES pellet lasting 1 week does not modify the hepatic glucuronyltransferase activity toward DES (5). Himaya et al. (8) did not observe variation in the enzymatic activity toward estradiol in rabbits treated for 8 days with estradiol. Chronic administration of DES (3.5 and 7 months) decreases the glucuronyltransferase activity; this effect is more obvious in animals treated for 7 months. This phenomenon has also been described in the case of estradiol (10).

The results obtained in the determination of glucuronyltransferase activity do not confirm Horning’s hypothesis because we have found that, in the presence of UDPGA, activity is 5 times higher in hamster kidney than in rat kidney. Although the prolonged treatment with DES decreases glucuronyltransferase, this behavior is general in other species and does not suppose a peculiarity in the case of the hamster. On the other hand, the decrease of activity with treatment is gradual, and after 7 months the activity even reaches approximately 50% of the initial level, an amount still considerably higher than that of the nontreated rat (5).

Chronic administration of DESGA has not produced renal tumors in the hamster. This fact seems to indicate that the tumorigenic activity of DES is due to DES itself and not to its metabolite, DESGA. The reaction product of β-glucuronidase

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activity toward DESGA is DES and that of the O-demethylase activity toward DESDME can be DES or DES monomethyl ether depending on whether the enzyme acts on 2 or 1 of the methoxy groups of the molecule. With the results obtained in vitro, we see that hamster liver produces 66 times more DES from DESGA than from DESDME. The different levels of DES formed from precursors DESGA and DESDME should make renal tumor production much more likely with DESGA treatment than with DESDME treatment. At first sight, the results obtained in vivo conflict with the predictions deduced from the results obtained in vitro. From our point of view, the main factor that prevents us from comparing, on the same basis, the production of renal tumors by DESGA and DESDME with the levels of the enzymes that act on them is the difference in the physicochemical properties of both compounds. DESGA is much less lipid soluble than DESDME and is absorbed from the site of injection and eliminated in the urine more quickly than DESDME.

The hepatic metabolism of DESGA and DESDME is also influenced by the physicochemical differences between them. DESDME easily traverses the cell membrane and DESGA does not; so DESDME gains access to the demethylase, and DESGA is to some extent confined to the extracellular fluid, being partially carried away from the liver by the circulation without gaining access to β-glucuronidase. Another part of the DESGA is excreted in the bile in an active transport process (14). The entrance of DESGA into a cycle of enterohepatic circulation (more or less closed according to the β-glucuronidase intestinal content) is dependent upon the mechanism of biliary excretion and carries with it the subsequent formation of DES. There are no references concerning the intensity of the mechanism of biliary excretion in the hamster.

Another matter to consider is the possibility of β-glucuronidase action in vivo. β-Glucuronidase levels in serum and bile are lower than in organs; on the other hand, the presence of saccharolactone in bile (4) and nondialyzable inhibitors in serum (3) has been described, which indicates that the enzyme of these fluids should contribute very little to the formation of free aglycone.

The role played in vivo by β-glucuronidase in organs is much discussed (4). It seems that the enzyme has a double localization, lysosomal and extralysosomal (mainly in the endoplasmic reticulum). In the intact cell, the primary role of β-glucuronidase would be in the endoplasmic reticulum, and in the lysosome the enzyme would be in a latent state and become functional only after rupture of the membranes and release in the medium (18).

The studies performed on the utilization of glucuronides administered parenterally indicate that, as in our case, they are very poorly metabolized, although in vitro the β-glucuronidase levels found in organs are high. Androsterone (16) and phenol (7) glucuronides are eliminated unchanged in the urine of man and rabbit, respectively. DESGA preserves only 5 to 10% of the estrogenic activity of DES when administered to rabbits (17); the 3-glucuronide of morphine has no pharmacological activity when injected into mice and dogs (20).

The low hepatic metabolism of DESDME presents the problem of whether the renal tumors produced are due to DESDME itself or to its metabolites, DES or DES monomethyl ether. It is difficult to imagine that DESDME with the active groups blocked and with an estrogenic activity by s.c. 45 times lower than that of DES (15) could produce renal tumors. Although, according to the results obtained in vitro, the hepatic metabolism of DESDME is very low, one must consider the fact that in our experiments, the dose of DES administered (equimolar with the DESDME one) was higher than the minimum oncogenic dose. Kirkman and Bacon (13) found that the minimum dose required for the induction of renal tumors was 0.03 mg daily by s.c. administration. It is possible that in our case the DES formed by the demethylation reaction falls within the minimum oncogenic dose and the longer latent period and the appearance of smaller tumors may be due to the lower levels of DES.

**REFERENCES**


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