ABSTRACT

The ability of vitamin C to inhibit induction of renal carcinoma by estrogens was tested in male Syrian hamsters in vivo. The animals received estrogen (estradiol or diethylstilbestrol) implants s.c. Hamsters which were continuously given vitamin C, administered in the drinking water for estradiol-treated or in the food for diethylstilbestrol-treated animals, were observed to develop renal carcinoma with a significantly lower incidence (10 of 33 animals with estradiol implants; 14 of 29 animals with diethylstilbestrol implants) than animals which did not receive vitamin C supplementation (16 of 23 animals with estradiol implants; 11 of 13 animals with diethylstilbestrol implants). Administration of vitamin C to estradiol-treated hamsters for only the first 3 months of the carcinogenesis experiment had no effect on tumor incidence, but vitamin C in drinking water for the last 3 months also lowered incidence. Vitamin C supplementation did not significantly alter the absorption of estrogen from the implant; it did not change the estrogenic effect on the hamsters nor did it significantly influence estrogen-dependent H-301 tumor cell growth. The results were taken as evidence for a mechanism of tumor induction via oxidation of estrogens to reactive metabolites capable of inducing kidney tumors.

INTRODUCTION

Synthetic or natural estrogens such as DES or 17β-estradiol implanted s.c. have been shown to induce (8) renal carcinoma in Syrian hamsters within 6 to 8 months. The mechanism of estrogen-induced carcinogenesis is still unknown. At this time, possibilities of hormonal imbalance or uncontrolled stimulation of cell proliferation have been discussed (6). It has also been suggested (12, 13) that reactive estrogen metabolites may interact with cellular macromolecules and thus initiate tumors. In experiments with 17β-estradiol, a semiquinone-quinone formed from catechol estrogens was suggested to be the reactive intermediate responsible for enzyme-mediated binding of 17β-estradiol to peptides, proteins (5, 15, 23), or DNA (18). DES quinone was found to be a reactive DES metabolite (12) which bound to DNA (13) and was therefore proposed to be a carcinogenic intermediate.

In the following study, the influence of vitamin C (L-ascorbic acid) on estrogen-induced renal carcinoma in the Syrian hamster was examined in an attempt to determine mechanistic details of hormonal tumorigenesis. If the formation of reactive metabolites was indeed a crucial event in tumor initiation by estrogens, it was expected that modulation of estrogen-induced neoplasms was possible by coadministration of vitamin C in vivo. The intracellular reduction of quinone-semiquinone intermediates by vitamin C in vivo, in analogy to reductions of synthetic DES quinone by vitamin C to a mixture of DES and (2) diethylstilbestrol (14), was expected to diminish the carcinogenic effects of administered estrogens. These studies were designed in a manner similar to the successful prevention by vitamin C of liver, lung, or stomach tumors induced by coadministration of amines and sodium nitrite [for review, see Mirvish (19)]. In those experiments, the mechanism of action of vitamin C was postulated (19, 25, 29) to proceed by reduction of nitrite and lead ultimately to prevention of i.g. nitrosation reactions, similar to prevention of nitrosamine or nitrosoureia formation in simple chemical model tests (19). If, on the other hand, hormonal carcinogenesis did not involve reactive carcinogenic estrogen metabolites (hormonal imbalance), vitamin C administration to estrogen-treated Syrian hamsters was not expected to alter kidney tumor frequencies.

The estrogen-induced Syrian hamster kidney tumor (8) was selected for attempts to modulate tumorigenesis by coadministration of vitamin C, because estrogen alone induced neoplasms, because of the high tumor incidence, and because of the estrogen dependence of the tumor for growth (8, 26). These characteristics of the cancer model were expected to be sensitive indicators of any effect of vitamin C in vivo. Furthermore, inhibition of tumorigenesis in the hamster kidney had been demonstrated (8) by coadministration of progesterone, testosterone, deoxyxycorticosterone acetate, and other agents.

MATERIALS AND METHODS

Animals. Male Syrian hamsters, 3 to 4 weeks of age, were purchased from Harlan/Sprague Dawley, Madison, Wisconsin. Hamsters were kept in groups of 5 animals/cage with food and water available ad libitum.

Chemicals. Vitamin C was a gift of Dr. Hemmige N. Bhagavan, Hoffmann-LaRoche Inc., Nutley, N.J. DES, cholesterol, and 17β-estradiol were purchased from Sigma Chemical Co., St. Louis, Mo. 2,4-Dideuterioestradiol was synthesized as described in the literature (21).

Diets. Purified hamster diet which did not contain any added vitamin C was obtained in pellet form from ICN Nutritional Biochemicals, Cleveland, Ohio. The same purified hamster diet, enriched with 1 g of vitamin C per kg of food, and a second batch, enriched with 2.5 g of vitamin C per kg of food, were purchased from the same company. The hamster food, in plastic bags, was stored in the dark at 5°. Three months after the beginning of the in vivo experiment, fresh diet with the same composition was purchased from ICN Nutritional Biochemicals.

Instrumentation. Extracts of pellet remains were analyzed using a Finnigan gas chromatograph/mass spectrometer, Model 3200, combined with a Finnigan Incos data system (6 ft x 2-mm glass column, packed with 3% OV-1 on Gas Chrom Q, 100/120 mesh, injector temperature 250°, column temperature 200–290°, 12°/min increase, 70 eV).

In Vivo Carcinogenesis. For the DES-induced carcinogenesis experiments, 45 male Syrian hamsters were divided into 3 groups of 15 animals/group. The first group received the vitamin C-free purified hamster diet. The second group received the same diet enriched with vitamin C (1 g/kg of food), and the third group was fed the purified diet enriched...
with vitamin C (2.5 g/kg of food). After 2 days, all animals received two 40-mg pellets implanted s.c. consisting of 90% DES and 10% cholesterol. Three months later, each animal received one more implant of the same weight and composition. After 172 days, each animal was weighed and killed by decapitation. Both kidneys were excised and placed in 10% formalin solution. From each kidney, 2 sections were prepared for histological examination. Sections were cut and examined by an unbiased pathologist without knowledge of the treatment received by the animals. Tumors were reported by Kirkman and Robbins (9) to originate at the proximal tubule and proliferate to nodules of several mm in diameter grossly visible on the surface of the kidneys. Therefore, microscopic foci within the kidney were considered small, while tumor foci were considered large when they were grossly visible. As reported by Kirkman and Robbins (9), renal neoplasms were histologically indistinguishable when induced by 17β-estradiol or DES. A gross examination of the kidneys for visible tumor nodules carried out by an unbiased observer prior to histology showed results comparable to those of the histological examination. Only the histological examination is reported here due to its inherent higher accuracy. Macroscopic and microscopic renal carcinoma foci were determined and tabulated. Three of the total of 45 animals were lost in the animal care facility or died of various causes.

For the 17β-estradiol-induced carcinogenesis experiment, a total of 120 male Syrian hamsters were used. In this experiment, vitamin C was administered in the drinking water, which was available ad libitum. The animal feed was vitamin C-free purified hamster diet. The drinking water was a solution of vitamin C (1% w/v) and sucrose (0.5% w/v) in reversed osmosis deionized water which was administered in glass bottles fitted with glass drinking tubes. Fresh solutions were prepared and given to the hamsters every 24 hr. A control group of 10 animals received vitamin C in the drinking water but was not supplied with estrogen. A second control group (10 animals) received neither 17β-estradiol nor vitamin C (drinking water, 0.5% solution of sucrose in deionized water). The positive control group (25 hamsters) was provided with one 31-mg implant consisting of 90% 17β-estradiol and 10% cholesterol but received only a solution of 0.5% sucrose in reversed osmosis deionized water for drinking (no vitamin C added). The experimental group (35 hamsters) was treated with 17β-estradiol implants in the same way as the positive control group but was given the vitamin C solution for the duration of the experiment. One more experimental group (20 animals) treated with 17β-estradiol implantations was allowed to drink the vitamin C solution from Day 1 to Day 92 of the experiment but received only sucrose solution thereafter. Another experimental group, pelletted with 17β-estradiol (20 hamsters), was treated with the sucrose solution instead but received drinking water containing vitamin C from Day 93 to the end of the experiment. All estrogen-treated animals were resupplied with 17β-estradiol implants (45-mg pellets; 90% 17β-estradiol: 10% cholesterol) 3 months after the beginning of the experiment. After 196 days, all animals were killed, and their kidneys were examined histologically as described above. Twelve animals of the total of 120 hamsters were lost in the animal care facility or died of various causes during the experiment. To determine any influence of vitamin C on the estrogenic effect of the 17β-estradiol implants, both testes were excised in addition to removal of the kidneys, and the testes were weighed. Weights of both testes are given in mg and are also expressed as percentage of total body weight.

17β-Estradiol Absorption. To determine the influence of vitamin C on absorption of 17β-estradiol from the pellets, the remaining pellet was recovered from 3 animals of the 17β-estradiol-treated (positive control) group and from 3 animals of the 17β-estradiol- and vitamin C-treated group. 2,4-Dideuterioestradiol (10 mg) was added as internal standard to the pellet remains from each hamster, and each mixture was extracted twice with 5 ml of warm methanol. The methanol solutions were dried in a stream of nitrogen, and the residues were trimethylsilylated using N,O-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co., Rockford, Il.) and dry pyridine (10:1, v/v) and analyzed by gas chromatography-mass spectrometry. The amounts of 17β-estradiol in the pellet remains from each hamster were calculated from the ratios of the molecular ions of trimethylsilylated 17β-estradiol and 2,4-dideuterioestradiol, m/z 416 and m/z 418, respectively. An average absorption rate was calculated by dividing the amount of total absorbed 17β-estradiol by the total number of exposure days, 196 days.

RESULTS

Influence of Vitamin C on DES-induced Carcinogenesis. Implantation of DES pellets into male Syrian hamsters resulted in a high incidence of renal carcinoma after approximately 5.5 months. A kidney tumor frequency of approximately 85% of the animal population after 172 days of exposure to DES implants matched well the high tumor incidence rates found earlier (8). Although vitamin C was administered to DES-treated hamsters at 2 different concentrations in the diet, results from the 2 groups were combined (Table 1) since no significant differences were found (14) between them. More than one-half of all vitamin C-treated animals were found free of tumors. A statistical evaluation of the tumor frequency data showed that coadministration of vitamin C resulted in a significant inhibition of DES-induced kidney tumors in male Syrian hamsters (p < 0.05). In a preliminary communication (14), results of the 2 groups were analyzed separately. The results of a gross visual examination and of kidney histology were shown to match closely.

Influence of Vitamin C on 17β-Estradiol-induced Carcinogenesis. Treatment of male Syrian hamsters with 17β-estradiol implants after approximately 6.5 months led to renal neoplasms in about 70% of the animals (Table 1). As reported earlier by Kirkman and Robbins (9), 17β-estradiol-induced tumors were histologically indistinguishable from DES-induced tumors. In contrast, coadministration of vitamin C (in drinking water) resulted in a tumor incidence of only 30%, less than one-half of that of animals not receiving vitamin C. Statistically, the reduction of the tumor incidence by administration of vitamin C was highly significant (p < 0.005). Furthermore, animals with vitamin C treatment had less severe kidney cancer. The total number of tumor foci per tumorous animals was significantly reduced, and the total number of large foci was lower. As expected, control groups of hamsters without estrogen treatment were completely free of tumors.

In 2 other experimental groups of 17β-estradiol-treated ham-
The same age. In Table 1, testes weights of hamsters are as a result of vitamin C treatment. It is possible that this test is statistically significant differences in testes weights were found hamsters have been reported (8) in experiments with significantly significant and probably cannot be used to justify the differences with these data due to the low number of animals examined in.

306 μg/day. Although a statistical analysis was not carried out with these data due to the low number of animals examined in each group, the differences in absorption do not appear to be significant and probably cannot be used to justify the differences in tumor frequency rates. Moreover, high tumor frequencies in hamsters have been reported (8) in experiments with significantly lower estradiol absorption.

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\[ \text{Av. body wt (g)} = \frac{(17\beta-estradiol, vitamin C (Days 93-196) \times 61.3) - \text{Remaining 17\beta-estradiol}}{196} \]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor bearing/total no. of animals (%)</th>
<th>Total no. of foci (^a)</th>
<th>Av. no. of tumors/tumor-bearing animal (^b)</th>
<th>Av. body wt (g)</th>
<th>Wt (mg)</th>
<th>% of body wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0/9 (0)</td>
<td></td>
<td></td>
<td>164 ± 19 (^c)</td>
<td>4070 ± 350</td>
<td>2.5</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>0/7 (0)</td>
<td></td>
<td></td>
<td>135 ± 10</td>
<td>3940 ± 290</td>
<td>2.9</td>
</tr>
<tr>
<td>17\beta-Estradiol</td>
<td>16/23 (70)</td>
<td>18</td>
<td>19</td>
<td>130 ± 17</td>
<td>214 ± 43</td>
<td>0.17</td>
</tr>
<tr>
<td>17\beta-Estradiol, vitamin C</td>
<td>10/35 (30)</td>
<td>4</td>
<td>9</td>
<td>128 ± 17</td>
<td>361 ± 17</td>
<td>0.17</td>
</tr>
<tr>
<td>17\beta-Estradiol (Days 1-92); 17\beta-Estradiol, vitamin C (Days 93-196)</td>
<td>9/19 (47)</td>
<td>5</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>17\beta-Estradiol, vitamin C (Days 1-92); 17\beta-Estradiol, [Days 93-196]</td>
<td>15/17 (86)</td>
<td>4</td>
<td>20</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DES</td>
<td>11/13 (85)</td>
<td>2</td>
<td>29</td>
<td>121 ± 11</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DES, vitamin C</td>
<td>14/29 (48)</td>
<td>3</td>
<td>47</td>
<td>128 ± 12</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^{a}\) Sum of all macroscopic or microscopic tumor foci determined by histopathological determination in this group of animals.

\(^{b}\) Total number of large and small tumor foci determined histopathologically (previous 2 columns) per number of animals with tumors.

\(^{c}\) Mean weight ± S.D.

\(^{d}\) Significantly different from positive control, at p < 0.005.

\(^{e}\) ND, not determined.

\(^{f}\) Significantly different from positive control, at p < 0.05.

Influence of Vitamin C on Also, any correction by vitamin C of hormonal status of mammals is limited. Any effect of vitamin C on pituitary- or thyroid-regulating hormone production or hormonal control is not known. Certainly, any correction by vitamin C of hormonal imbalance or malfunction of hormonal control has never been reported. In steroid-forming organs, however, elevated concentrations of vitamin C are known (7) to inhibit 11\beta-hydroxylation of steroids. Therefore, a decrease was observed (7, 24) in corticosteroid synthesis in adrenal tissue in vitro in the presence of vitamin C. Furthermore, ovarian or testicular conversion of cholesterol to steroid hormones was partially or completely inhibited by administration of vitamin C, metyrapone, or aminoglutethimide (3, 27). These effects were not an influence of vitamin C at the level of hormonal control but were explained as an inhibition of cytochrome P-450 enzymes involved in steroid metabolism (3, 27).

Intake of vitamin C has been observed (1, 2, 20) to increase the plasma concentration of exogenous estrogens in humans. Vitamin C was suspected to compete with estrogens for sulfation. Therefore, the increase in plasma concen-
Chart 1. Estradiol-dependent growth of H-301 tumor cells in male Syrian hamsters. On Days 12, 20, and 25 after inoculation of cells, diameters of growing tumors were measured (left). Immediately after the last measurements, animals were killed, and the masses of excised tumors were determined (right). Control animals (top) did not receive vitamin C.

The concentration of estrogen was suggested (1, 2, 20) to be due to slowed metabolic clearance. In view of these findings, it is possible that vitamin C administration to estrogen-treated male Syrian hamsters resulted in an increase in circulating 17β-estradiol (or DES) compared to animals without vitamin C supplementation. Any effect of vitamin C on steroidogenesis in the adrenals or in the atrophied testes of estrogen-treated male hamsters is not considered to be sufficiently large to serve as an explanation of decreased carcinogenesis. The significant reduction in estrogen tumorigenesis in male Syrian hamster by coadministration of vitamin C is therefore suggested to be evidence against a mechanism of hormonal imbalance or uncontrolled cell proliferation induced by exogenous estrogen, at least in this tumor model. In addition, the inability of vitamin C to significantly influence 17β-estradiol-dependent H-301 tumor growth in vivo is taken as an indication of an endocrine status unaltered by vitamin C and of a lack of any effect of vitamin C on the 17β-estradiol-induced factors (26) responsible for H-301 growth.

Instead, a mechanism of estrogen-induced cell transformation via metabolic oxidation of 17β-estradiol (or DES) to reactive, carcinogenic metabolites is proposed (13, 18). DES quinone, formed by enzyme-catalyzed oxidation of DES, was suggested (12) to be a carcinogenic DES metabolite, since it bound to DNA without further enzyme mediation. Similarly, enzyme-mediated oxidation of 2- or 4-hydroxyestradiol to quinones-semiquinones was proposed to occur prior to observed binding of estrogen to proteins, peptides (15, 18), or DNA (18). Based on these in vitro experiments, binding of estrogen metabolites to DNA or other important cellular macromolecules was postulated (11, 18) to be the tumor-initiating event. The lowered incidence of kidney tumors as a result of vitamin C administration may be due to a continuous chemical reduction of quinone-semiquinone metabolites to harmless metabolic intermediates in vivo. Another possible mechanism of action of ascorbic acid may be the reduction of other highly reactive endogenous compounds such as superoxide radicals which may be formed during metabolism of estrogens and which may be the final DNA-damaging species. Superoxide radicals as reactive intermediates have been discussed by Trush et al. (28).

Indeed, the observed (4, 17, 22) morphological and neoplastic transformation of Syrian hamster fibroblasts or of BALB/c 3T3 cells in culture has been correlated to the ability of these cells to metabolically activate the administered estrogens.

The results obtained from the exposure of 17β-estradiol-treated Syrian hamsters to vitamin C for a limited period of time (Table 1) allow a refinement of the proposed mechanism of tumor induction. A lowering of the kidney tumor frequency in 17β-estradiol-treated animals was partially successful only when
vitamin C was administered during the last 3.5 months of the experiment, but not with vitamin C intake during the first 3 months after 17β-estradiol implantation. These results suggest that cellular transformation by carcinogenic 17β-estradiol metabolites may take place only after an initial period of hormonal preparation of the cellular environment for the carcinogenic event; estrogens in the hamster kidney may first activate metabolizing enzymes which in turn may be responsible for increased concentrations of quinone-semiquinone metabolites. Furthermore, the receptor status of hamster kidney cells (10) may be altered by estrogen administration to allow uptake of catechols or quinones into the cell nucleus. It is thus postulated that only after these and other hormonally induced biological or biochemical changes may hamster kidney cell transformation take place.

ACKNOWLEDGMENTS

The authors are indebted to Dr. David A. Sirbasku for making available the H-301 tumor cell line and Judy Roscoe for growing the cells needed for the tumor growth experiment. Tseng-Ying Fan’s skilful operation of the mass spectrometer after these and other hormonally induced biological or biochemical changes may hamster kidney cell transformation take place.

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Inhibition of Estrogen-induced Renal Carcinoma in Syrian Hamsters by Vitamin C

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