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Chemoprevention by Dietary Dehydroepiandrosterone Against Promotion/Progression Phase of Radiation-induced Mammary Tumorigenesis in Rats

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When pregnant rats received whole body irradiation with 260 cGy γ-ray at day 20 of pregnancy, and were then implanted with a diethylstilbestrol (DES) pellet for an experimental period of 1 year under feeding of a control diet, a high incidence (96.2%) of mammary tumors was observed. Administration of dietary 0.6% dehydroepiandrosterone (DHEA) together with DES implantation significantly decreased the incidence (35.0%) of mammary tumors. The first appearance of palpable tumors in the DHEA-fed group was 4.5 months later than that in the control group. For clarification of the mechanism of the chemopreventive action, we measured hormone levels in the serum of DHEA-fed rats. In the DHEA diet rats, the concentration of estradiol-17β exceeded, by approximately 6-fold, that in the control rats, while the levels of progesterone and prolactin were decreased by 30 and 45%, respectively. Interestingly, DHEA feeding prevented DES-induced hypertrophy of pituitary glands and DES-induced high level of prolactin in pituitary glands detected by immunohistochemical studies, but stimulated the development of mammary glands more than that in control rats treated with DES alone. These findings suggest that DHEA has a potent preventive activity against the promotion/progression phase of radiation-induced mammary tumorigenesis. The mechanism of chemoprevention by change of endocrinological environment is discussed.

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INTRODUCTION

DHEA is a major secretory steroid of the adrenal glands and its biological significance is known to be as a precursor steroid in the biosynthesis of androgens and estrogens. It has been reported that low plasma level of DHEA may be associated with an increased risk of breast cancer in women [1]. In rodents, the oral administration of DHEA inhibits the appearance of spontaneous mammary cancer in female C3H(A^{vy}/a) mice [2] and reduced the incidence of *N*-nitroso-*N*-methylurea (NMU) [3] and 7,12-dimethylbenz(a)anthracene (DMBA) [4] induced mammary cancer in Sprague–Dawley rats. Also, dietary DHEA suppresses chemical carcinogenesis in skin [5], lung [6], liver [7, 8], thyroid [8] and pancreas [9] of experimental animals. The mechanism of the chemopreventive action of DHEA remains uncertain. On the other hand, DES, a synthetic estrogen, promotes the development of rat mammary tumors initiated by γ-rays [10]. Previous studies in our laboratory demonstrated that DES acts on radiation-initiated mammary cells by binding directly with estrogen receptor (ER) and/or by stimulation of prolactin secretion from the pituitary glands [11]. The present study was designed to evaluate the anti-carcinogenic activity of DHEA against DES-dependent promotion/progression of radiation-induced mammary tumors. Furthermore, the

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mechanism(s) of the chemopreventive activity of DHEA is discussed from oncological and endocrinological viewpoints.

EXPERIMENTAL

Materials

[2,4,6,7-^{3}H]estradiol-17β (sp. act. 4.0 TBq/mmol) and [17α-methyl-^{3}H]17α-methyl-17-propionylestra-4,9-dien-3-one (R5020) (sp. act. 3.0 TBq/mmol) were purchased from DuPont/NEN Research Products (Boston, MA). Estradiol-6-[(10-carboxymethyl)-oximino-(2-[^{125}I]iodohistamine)] (sp. act. 74 TBq/mmol) was purchased from Amersham (Aylesbury, U.K.). DES and DHEA in powder form were obtained from Sigma (St Louis, MO), and pellets were prepared from a mixture containing 3 mg of DES and 27 mg of cholesterol in a medical grade Silastic tube (Dow Corning Co. Midland, MI; 1.98 mm inner diameter × 3.18 mm outer diameter) [12]. Diet containing 0.6% (w/w) DHEA was prepared in biscuit form by Funabashi Farm (Chiba, Japan). A basal diet of the same form (MB-1) was used for the control experiments.

Animals and treatment

Wistar–MS rats, bred in this Institute, were kept at 23 ± 1°C in a controlled environment (14 h light–10 h dark). They received water and food *ad libitum*. Forty-six pregnant rats received whole body irradiation with 260 cGy γ-rays (17 cGy/min) from a ^{60}Co source at day 20 of pregnancy (the presence of a vaginal plug denoting day 1) and were divided into two groups after weaning. Serving as the control group, 26 rats were fed a basal diet and were then implanted with a DES pellet at 20 days after weaning. Twenty rats were fed diet containing 0.6% DHEA immediately after weaning and received a DES pellet at 20 days after termination of nursing (Fig. 1). The pellets were replaced every 8 weeks. The rate of release of DES from the pellets was approx. 0.38 ± 0.01 μg/day [11]. The rats were observed for 1 y and screened for palpable mammary tumors. When tumors larger than 2 cm in diameter were detected, the rats were sacrificed by CO_2 asphyxiation and the tumors were removed.

Histological examinations of whole mount mammary gland specimens

The entire inguinal mammary glands after the 1 y experimental period were dissected from the inner surface of the skin, retaining as much of the connective tissue as possible, spread and dried slightly on filter paper. After fixation in 10% neutral buffered formalin and defatting in acetone, the specimens were stained with alum carmin and stored in Cedar oil [13].

Immunohistochemical detection of prolactin in pituitary glands

Pituitary glands were fixed in Bouin's solution without acetic acid for 4 h. The tissues were dehydrated and embedded in paraffin. Sections (4 μm in thickness) of the pituitary glands were deparaffinized, and immunohistochemical staining was performed by streptavidin–biotin method (Histofine SAB-PO(R9) kit, Nichirei Co. Tokyo) using anti-rat prolactin S-9 antiserum (NIDDK) supplied by the National Hormone and Pituitary Program (Baltimore, MD). Immunoreactive tissues were visualized by horse radish peroxidase using 3,3′-diaminobenzidine. The specificity of staining was confirmed by use of non-immunized normal rabbit serum. The sections were counterstained lightly with hematoxylin.

Assay of steroid receptors

The tumor tissues were homogenized with 10 mM Tris–HCl buffer (pH 7.4) containing 1.5 mM EDTA-Na and 1 mM dithiothreitol. Homogenates were centrifuged at 105,000 ***g*** for 1 h at 4°C, and the obtained cytosol fraction was used for assay of the receptors. ER and progesterone receptor (PgR) in the cytosol fraction were analyzed by the dextran-coated charcoal method using [2,4,6,7-^{3}H]estradiol-17β and [17α-methyl-^{3}H]R5020, respectively, as radio-ligands [14, 15].

Measurement of hormones

A blood sample, collected from each rat by cardiocentesis under anesthesia, was allowed to clot and centrifuged to obtain serum. The sera were frozen immediately and stored at −80°C until the assay was started. Concentrations of prolactin, LH, FSH and

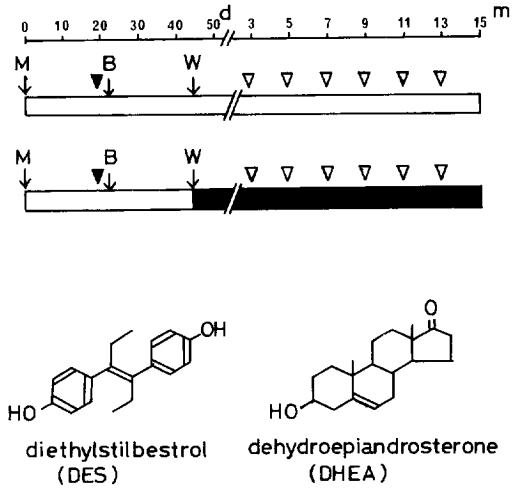

Fig. 1. Experimental protocol and chemical structures of the compounds used in this study. M, mating; B, birth; W, weaning; Closed arrowhead, whole body irradiation with 260 cGy γ-rays at day 20 of pregnancy; open arrowhead, implantation with DES pellet; open bar, control diet (MB-1); closed bar, diet containing 0.6% DHEA; d, day-old; and m, month-old.

TSH in each serum sample were determined with NIDDK radioimmunoassay kit (the National Hormone and Pituitary Program, Baltimore, MD). Estradiol-17β was measured by a modified method of Watanabe *et al.* [16]. The serum concentrations of progesterone, 11-deoxycorticosterone (DOC), dehydroepiandrosterone (DHEA), DHEA-sulfate (DHEA-S), free cholesterol, cholesterol ester and triglyceride were assayed by commercially available kits.

Iball's index and statistical analysis

Iball's index was calculated as follows: the ratio of incidence (%) to the average latency period in days was multiplied by 100 [17]. Statistical analyses were conducted by Fisher's exact probability test for incidence, and Student's t-test for the level of significance of differences between pairs of mean values for body and organ weights and hormone concentrations.

RESULTS

Biological effects of long-term treatment with DHEA

The body weight was significantly decreased in all rats by treatment with DES, and marked loss of weight was observed in the rats fed DHEA diet (Table 1). The body weight in control rats was reduced temporarily after weaning but then recovered to the starting value 10 days later. However, the reduced weight in DHEA-fed rats declined further upon implantation of DES pellets, and then decreased slowly during the 1 y period (Fig. 2). Both groups of rats consumed similar amounts of food throughout the experiment. The liver weight of the DHEA-fed rats was 2.5-fold greater than that of the control animals ($P < 0.01$). In addition, we found that the liver of rats treated with DHEA-containing diet acquired a deep mahogany color while that of the control rats did not. The causes of the color change in the liver could not be determined. DHEA also caused increased weight of the uterus ($P < 0.01$) and ovaries ($P < 0.01$). The control rats implanted with DES had enlarged pituitary glands which presented macroscopically friable hemorrhagic hypertrophy, while DHEA feeding prevented DES-induced hypertrophy of the pituitary glands ($P < 0.05$).

Table 1. *Biological effects by long-term administration of DHEA*

	Control diet (n)	0.6% DHEA diet (n)	Difference
Initial body weight* (g)	286.3 ± 6.0 (10)	294.0 ± 14.5 (12)	NS
Final body weight† (g)	225.5 ± 6.1 (10)	176.8 ± 9.9 (12)	$P < 0.01$
Uterus† (g)	0.78 ± 0.05 (10)	2.33 ± 0.16 (7)	$P < 0.01$
Ovaries† (mg)	78.1 ± 8.9 (10)	265.9 ± 85.0 (7)	$P < 0.01$
Liver† (g)	10.5 ± 0.71 (10)	22.3 ± 1.81 (8)	$P < 0.01$
Pituitary† (mg)	25.1 ± 3.51 (8)	14.9 ± 1.58 (6)	$P < 0.05$

Each value represents the mean ± SEM.
*At time of weaning (see Fig. 1).
†Final body weight and organ weights were measured at the end of the experiments.

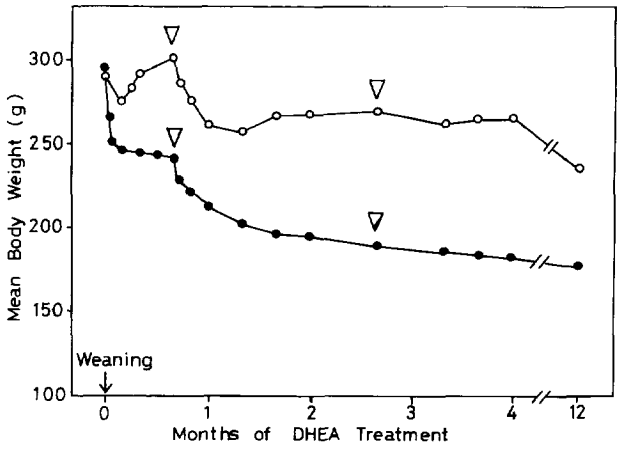

Fig. 2. Change in body weight during dietary DHEA administration. Twenty rats (●) were fed 0.6% DHEA-containing diet starting immediately after weaning, and then received a DES-pellet (▽) at 20 days after weaning. Twenty six rats (○) which served as controls were fed a basal diet (MB-1) and were implanted with a DES pellet at 20 days after weaning. The DES pellets were replaced every 8 weeks.

Histology of mammary gland whole mount preparations

Whole mounts of the entire inguinal mammary glands in DHEA-fed rats were prepared at the terminus of the experiment in order to examine the degree of development and differentiation of the glands. In irradiated rats fed the control diet, the mammary glands showed some alveolar buds due to the DES pellet [Fig. 3(a)]. Administration of dietary DHEA together with DES implantation in the irradiated rats augmented the extensive lobuloalveolar growth of mammary glands [Fig. 3(b)].

Hormone levels in serum

DHEA administration for 1 y was reflected in elevated serum DHEA and DHEA-S levels (Table 2). DHEA-fed rats showed 100-fold higher levels of serum DHEA then that of control rats ($P < 0.05$), and 575-fold higher serum DHEA-S levels ($P < 0.01$). Compared with control rats, DHEA-fed rats showed a 6-fold increase ($P < 0.05$) in serum estradiol-17β concentration, but a 70% reduction ($P < 0.01$) in progesterone. The prolactin concentration was reduced significantly ($P < 0.05$) in rats fed the DHEA diet, but serum concentrations of LH and FSH were almost the same as those in the control rats. Feeding of DHEA also resulted in reduction of serum free cholesterol ($P < 0.05$) and triglyceride ($P < 0.05$), but not of cholesterol ester.

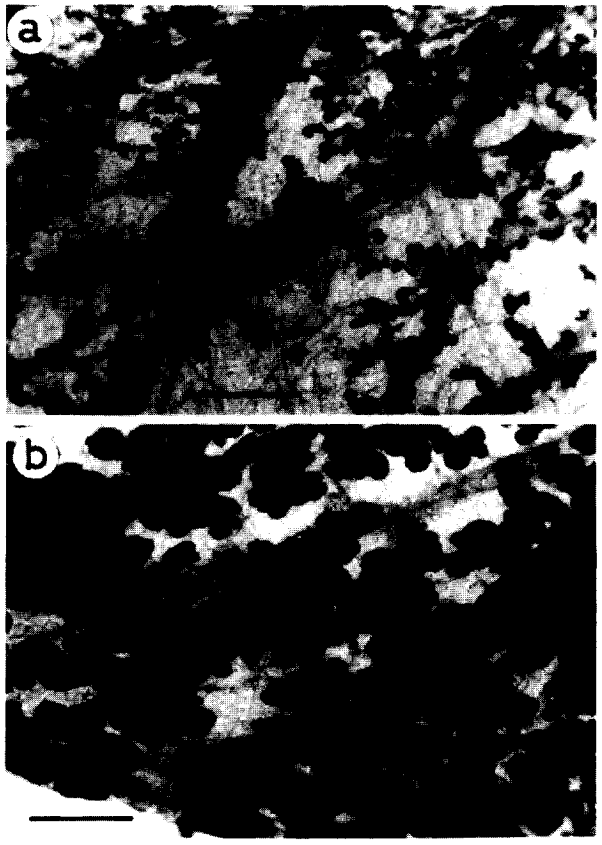

Fig. 3. Whole mount observation of inguinal mammary glands of rats. (a) DES-treated rats fed control diet (MB-1). (b) DES-treated rats fed diet containing 0.6% DHEA. Scale bar of each panel corresponds to 1 mm.

Prolactin in pituitary glands

Since DHEA inhibited the elevated level of circulating prolactin in irradiated rats implanted with DES, the immunohistochemical expression of prolactin in the pituitary glands of DHEA-treated rats was compared with that in the controls. Prolactin was detected in numerous pituitary cells of rats treated with DES [Fig. 4(a and c)]. On the other hand, DHEA caused atrophy of prolactin producing cells, and suppressed the DES-induced elevation of pituitary gland prolactin [Fig. 4(b and d)]. This was consistent with the serum concentration of prolactin (Table 2).

Effects of dietary DHEA on development of mammary tumors

Of the 26 pregnant rats that received whole body irradiation with 260 cGy γ-rays at day 20 of pregnancy and were then implanted with DES pellet under feeding of the control diet, 25 (96.2%) developed mammary tumors during the 1 y period (Table 3). The administration of dietary 0.6% DHEA together with DES implantation in rats irradiated in late pregnancy significantly decreased the total incidence (35.0%) of mammary tumors for the 1 y period ($P < 0.01$). However, hepatocarcinomas were found in three (15.0%) out of the 20 rats fed DHEA for up to 1 y (not significant compared with the control). There were no significant differences between the groups in the average of the latency period until appearance of mammary tumors or the number of mammary tumors per tumor-bearing rat. Iball's index for overall tumor development in the DHEA-fed rats was one-third of that in the control rats. As shown in Fig. 5, the first appearance of palpable tumors was delayed by approx. 4.5 months in the DHEA-fed group compared with that in the control group. The cumulative tumor incidence in the DHEA-fed group was significantly lower ($P < 0.05$) than that in the controls after 7.2 months.

Receptor for steroid hormone in mammary tumors

The mammary tumors (1 g) were homogenized, and the ER and PgR in the cytosol fraction were analyzed with a Scatchard plot. The results are shown in Table 4. Many (72.7%) of the mammary tumors in the rats under the control diet expressed ER(+)PgR(+), but no ER(−)PgR(−) tumor was observed. On the other hand, PgR(+) was observed in all tumors obtained from the group treated with DHEA.

DISCUSSION

Our results strongly suggest that DHEA has chemopreventive action against the DES-dependent promotion/progression phase of radiation-induced

Table 2. Effects of DHEA treatment on hormones, cholesterol and triglyceride in rat serum

Substance	Control diet (n)	0.6% DHEA diet (n)	Difference
Estradiol-17β (pg/ml)	6.06 ± 1.82 (7)	37.7 ± 10.2 (5)	$P < 0.05$
Progesterone (ng/ml)	47.6 ± 7.8 (8)	13.5 ± 2.3 (8)	$P < 0.01$
DOC (ng/ml)	103.5 ± 8.4 (3)	113.3 ± 63.5 (5)	NS
DHEA (ng/ml)	1.00 ± 0.23 (5)	100.0 ± 35.1 (5)	$P < 0.05$
DHEA-S (ng/ml)	32.2 ± 2.9 (5)	18,400 ± 1400 (5)	$P < 0.01$
Free cholesterol (mg/dl)	20.7 ± 3.2 (7)	8.4 ± 0.9 (5)	$P < 0.05$
Cholesterol ester (mg/dl)	62.3 ± 13.0 (7)	51.4 ± 3.9 (5)	NS
Triglyceride (mg/dl)	134.9 ± 16.0 (7)	88.8 ± 9.4 (5)	$P < 0.05$
LH (ng/ml)	0.31 ± 0.04 (8)	0.53 ± 0.12 (5)	NS
FSH (ng/ml)	6.04 ± 0.78 (8)	6.47 ± 0.49 (9)	NS
TSH (ng/ml)	2.60 ± 0.11 (5)	7.29 ± 1.08 (6)	$P < 0.01$
Prolactin (ng/ml)	487 ± 59 (8)	276 ± 45 (8)	$P < 0.05$

Each value represents the mean ± SEM.

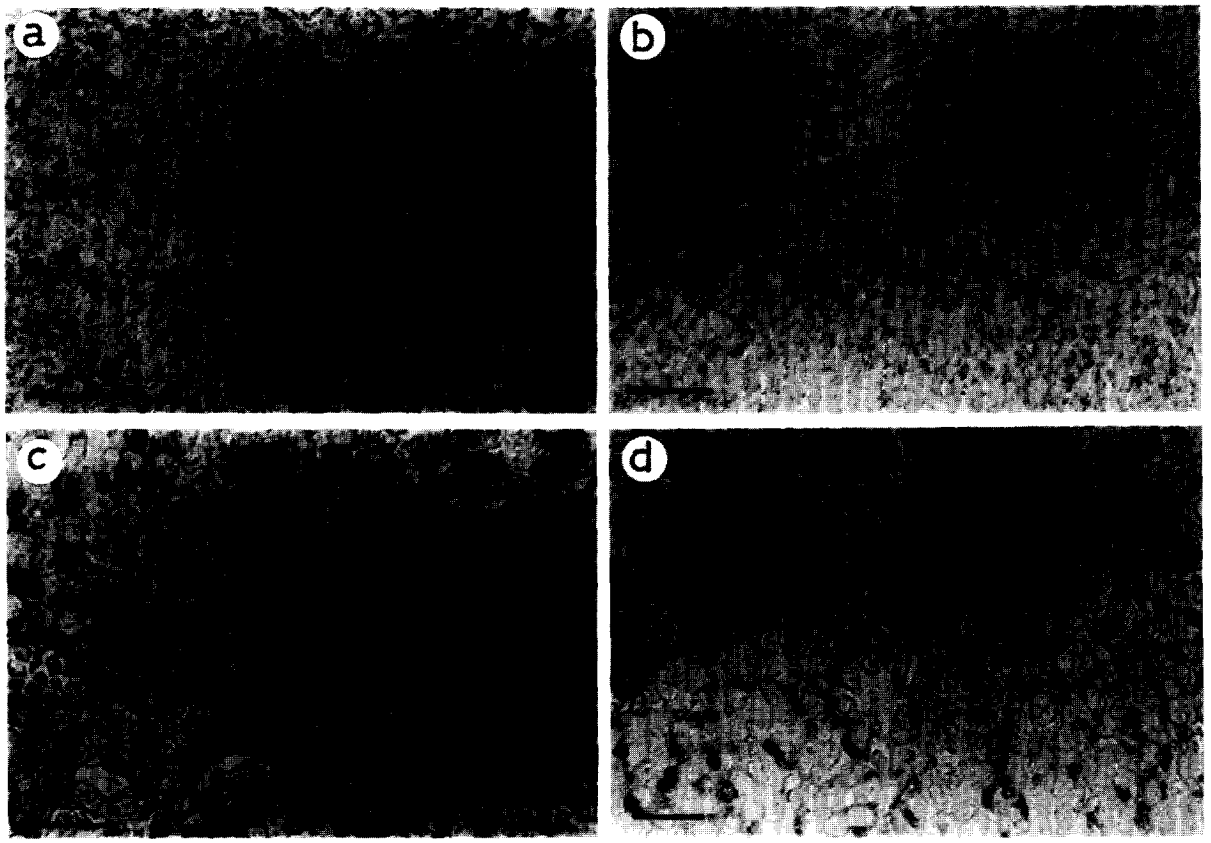

Fig. 4. Immunostaining of prolactin in pituitary glands of rats treated with DHEA. (a) DES-treated rats fed control diet (MB-1) and (b) DES-treated rats fed diet containing 0.6% DHEA. The scale bar on each panel corresponds to 100 μm. (c) and (d) High magnification of (a) and (b), respectively. The scale bar on each panel corresponds to 20 μm.

mammary carcinogenesis. The observed anti-carcinogenicity of DHEA is specific to some action of the steroid. DHEA reduced the growth of DMBA-induced mammary tumors in normal rats [4, 18], but stimulated that in ovariectomized rats [18]. DHEA also inhibited estrogen-induced proliferation of the MCF-7 human breast cancer cell line, but stimulated cell growth in estrogen-free medium [19]. DHEA is metabolized mainly to androst-5-ene-3β,17β-diol by 17β-hydroxysteroid dehydrogenase in the mammary glands in rats [20, 21]. Since DHEA itself has an extremely low relative binding affinity for ER [22], binding of the metabolite to ER is well known [23]. Our results clearly demonstrated that the DES-dependent promotion of radiation-induced mammary tumorigenesis is reduced by dietary DHEA. This observation is consistent with the possibility that androst-5-ene-3β,17β-diol derived from dietary DHEA competes with DES for

Table 3. Effects of DHEA treatment on DES-dependent progression of radiation-induced mammary tumors

	Control diet	0.6% DHEA diet	Difference
No. of rats used	26	20	
No. of rats with tumors	25	7	
Tumorigenesis (%)	96.2	35.0	$P < 0.01$
No. of tumors	44	12	
Latency period (months)	8.2 ± 0.6	9.1 ± 0.5	NS
Iball's index	39.1	12.8	
No. of tumors per tumor-bearing rat	2.1 ± 0.4	2.1 ± 1.1	NS

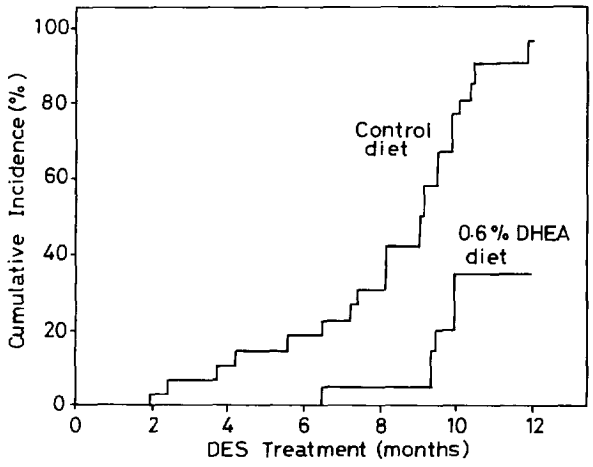

Fig. 5. Cumulative incidence of development of mammary tumors in irradiated rats treated with DES and DHEA. The tumor incidence in DHEA-fed rats was significantly lower ($P < 0.05$) than that in the control rats after 7.2 months.

Table 4. Receptor analysis in mammary tumors

Diet	No. of tumors tested	ER(+) PgR(+)	ER(+) PgR(−)	ER(−) PgR(+)	ER(−) PgR(−)
Control diet	11	8	2	1	0
0.6% DHEA diet	5	3	0	2	0

high-affinity intracellular binding sites for estrogen, thus reducing the promotion activity of DES.

Long-term administration of DHEA causes marked hypertrophy (3.4-fold) of ovaries. The estradiol-17β concentration in the serum of DHEA-fed rats was significantly increased (6.2-fold) compared with that in the controls, perhaps because administered DHEA may be converted to estradiol-17β *in vivo*. On the other hand, a low level (28% of the control value) of progesterone was observed in rats treated with DHEA. DHEA is a substrate for Δ^5-3β-hydroxysteroid dehydrogenase coupled with 5-ene→4-en-isomerase in the ovaries which also catalyzes conversion of pregnenolone to progesterone [24]. The reduced concentration of serum progesterone might be due to substrate inhibition of the Δ^5-3β-hydroxysteroid dehydrogenase coupled with 5-ene→4-en-isomerase by excess DHEA. The prolactin concentration of the serum and pituitary glands and the weight of the pituitary glands were significantly reduced in spite of enhancement of serum estradiol-17β level by long-term administration of DHEA. Since DHEA has a weakly androgenic activity, it may act competitively with estradiol-17β in the pituitary glands.

DHEA feeding results in peroxisomal proliferation in the liver [25] and induces hepatocarcinomas [26]. The induction of peroxisomal enzymes leads to an increase of oxidative stress by overproduction of H_2O_2 during activation of β-oxidation of fatty acids [25, 27]. Onkumar and Ramasarma [28] have reported that H_2O_2 may inactivate HMG-CoA reductase before its degradation by catalase in the cells. HMG-CoA reductase catalyzes a rate limiting reaction for biosynthesis of farnesyl-pyrophosphate and cholesterol. Inactivation of HMG-CoA reductase may cause suppression of isoprenylation of $p21^{ras}$, which is a critical step in the cell-transforming activity of oncogenic *ras* proteins [29]. In this context, inhibition of post-translational processing of $p21^{ras}$ by DHEA may contribute to its chemopreventive effects. Recently, Schulz and Nyce [30] have reported a mechanism of chemoprevention of colonic adenocarcinoma involving DHEA-dependent inhibition of $p21^{ras}$ isoprenylation.

HMG-CoA reductase requires 2 moles of NADPH for each mole of mevalonate produced. Since DHEA is a potent noncompetitive inhibitor of glucose-6-phosphate dehydrogenase [31], one of the rate-limiting enzymes for extramitochondrial NADPH production, DHEA-mediated inhibition of $p21^{ras}$ isoprenylation might occur due to depletion of NADPH required for HMG-CoA reductase activity. Conditions of reduced NADPH availability may also be responsible for the antiobesity action of DHEA.

Glutathione *S*-transferase is an enzyme that catalyzes cellular detoxification of electrophilic compounds, and inactivation of DES by glutathione *S*-transferase has previously been suggested [32]. DHEA, when administered as a supplement to the diet in rats, produces alterations in the relative concentrations of glutathione *S*-transferase in the liver [33]. Since irradiated rats receiving DES were simultaneously administered DHEA in these experiments, the promotion activity of DES may be rapidly reduced by the enhanced activity of glutathione *S*-transferase, and thus chemoprevention of mammary tumors may be suggested.

In conclusion, these findings indicate a protection effect of DHEA against the DES-dependent promotion/progression of radiation-induced mammary tumors in rats by a multi-function chemopreventive mechanism.

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