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2-Difluoromethylornithine and Dehydroepiandrosterone Inhibit Mammary Tumor Progression but not Mammary or Prostate Tumor Initiation in C3(1)/SV40 T/t-antigen Transgenic Mice[1]

Jeffrey E. Green,[2] Masa-Aki Shibata,[3] Eiko Shibata, Richard C. Moon, Miriam R. Anver, Gary Kelloff, and Ronald Lubet

Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, Bethesda, Maryland 20892 [J. E. G., M-A. S., E. S.]; Surgical Oncology Department, University of Illinois College of Medicine, Chicago, Illinois [R. M.]; Pathology-Histotechnology Laboratory, Science Application International Corporation, National Cancer Institute, Frederick, Maryland 21702 [M. R. A.]; and Division of Cancer Prevention, National Cancer Institute, Rockville, Maryland 20892 [G. K., R. L.]

ABSTRACT

Female transgenic mice that express SV40 T/t antigens under the regulatory control of the rat C3(1) gene spontaneously develop multifocal mammary lesions that predictably evolve into invasive, hormone-independent carcinomas, whereas male mice are prone to develop prostate cancer. Chemopreventive agents were administered to female C3(1)/SV40 large T-antigen mice from 7 to 19 weeks of age, during which time the mammary lesions developed and progressed to invasive carcinomas. No significant differences in the numbers of preinvasive mammary intraepithelial neoplasia lesions (histologically similar to human ductal carcinoma *in situ*) were observed after 2 or 8 weeks of treatment between mice receiving either vehicle alone, dehydroepiandrosterone (DHEA), or 2-difluoromethylornithine (DFMO). However, a dose-response reduction in invasive carcinoma growth was observed for both DFMO, an inhibitor of ornithine decarboxylase, and DHEA, the primary steroid precursor to both androgens and estrogens in primates. Despite unaltered expression of the transgene, tumor incidence was reduced approximately 20% by DFMO (8000 mg/kg) and 30% by DHEA (4000 mg/kg; $P < 0.05$). Tumor multiplicity was reduced by ~50% by both DFMO and DHEA ($P < 0.05$). DFMO had a dose-dependent effect on total tumor burden, which was reduced by 25% at low doses (4000 mg/kg) and 70% at high doses (8000 mg/kg). DHEA reduced tumor burden by 50% and 66% at low (2000 mg/kg) and high (4000 mg/kg) doses, respectively. Interestingly, despite its inhibitory effects on tumor development, DHEA caused a dose-dependent increase of serum estradiol levels that we have previously shown to increase mammary tumor formation in this model. No effect on the development of the prostate cancer precursor lesions (prostate intraepithelial neoplasia) was observed when mice were treated with DHEA, DFMO, tocopherol acetate, selenomethionine, or 9-*cis*-retinoic acid, although the effects on late-stage prostate cancer development were not determined. These results demonstrate that despite the expression of the highly transforming C3(1)/SV40 large T-antigen transgene, this transgenic model can be used to study the effects of chemopreventive agents on mammary cancer progression. The tumor-inhibitory effects of DHEA and DFMO on mammary cancer growth appear to occur after the development of preinvasive lesions, suggesting that these agents inhibit tumor progression but not initiation.

INTRODUCTION

A vast body of work examining potential chemopreventive agents in chemically induced rat mammary tumor models using 7,12-dimethylbenz(*a*)anthracene or MNU[4] has been performed (1). Several rat mammary tumor models share characteristics that are similar to human breast cancer including certain histopathological features (1), parallel changes in the expression of several genes involved in oncogenesis (2), and responses to known chemopreventive agents including tamoxifen, raloxifene, and various aromatase inhibitors (3–5). The inhibition of tumor development in the rat models by various hormonal manipulations reflects the fact that these tumors are hormonally responsive. Almost all of these chemically induced mammary tumors will regress even after the development of palpable lesions if the rats are ovariectomized (1). These models have been particularly useful in developing agents that suppress tumor development and growth by interfering with normal hormone signaling pathways. Thus, these models may be most useful for studying agents that might effect ER-positive, hormone-dependent human breast cancer.

A major question facing the field of chemoprevention is the identification of agents that will prevent the development or progression of human ER-negative, hormone-independent tumors. In this regard, mouse models of mammary carcinogenesis may be particularly applicable because most genetically engineered mouse models for mammary cancer appear to be hormone independent and do not respond to ovariectomy. The C3(1)/Tag transgenic mouse model for mammary and prostate cancer uses the 5′-flanking region of the C3(1) component of the rat prostatein protein to direct the expression of Tag and small t antigens from the SV40 early region (6). Tag has been shown to bind to and functionally inactivate two important tumor suppressor genes, p53 and Rb (7, 8), which are often deleted or mutated in human breast and prostate cancers (9, 10). Tag is particularly useful in the modeling of cancer in mice because it inactivates the function of multiple members of the Rb family, which appears to be critical for abrogating tumor suppressor function in the mouse. Thus, whereas Tag may not be etiologically related to these human cancers, Tag appears to subvert molecular pathways in these transgenic tumor models that also occur in the human cancers.

Female mice bearing this transgene develop mammary tumors by 5 months of age that follow a very predictable time course of lesion development. Low-grade MIN lesions develop after 8 weeks of age, progress to high-grade MIN (similar to human ductal carcinoma *in situ*) by 14 weeks of age, and advance to invasive adenocarcinomas at about 17 weeks of age (11, 12). The mammary adenocarcinomas that develop in this model appear to demonstrate very limited hormonal responsiveness. Recently, we have demonstrated that ovariectomy or treatment with tamoxifen has minimal effects on the development of grossly palpable tumors (13). In addition, expression of ER-α is lost during tumor progression, providing a potential mechanism for hormone independence of these mammary carcinomas. Additional molecular alterations have been identified that occur during tumor progression in this model (14, 15). We have recently demonstrated that 9-*cis*-RA suppresses mammary tumorigenesis in this transgenic model (16).

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[2] To whom requests for reprints should be addressed, at Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, NIH, Building 41, Room C629, 41 Library Drive, Bethesda, MD 20892. Phone: (301) 435-5193; Fax: (301) 496-8395; E-mail: jegreen@nih.gov.

[3] Present address: Osaka Medial College, Osaka 569-8686, Japan.

[4] The abbreviations used are: MNU, *N*-methyl-*N*-nitrosourea; DFMO, 2-difluoromethylornithine; DHEA, dehydroepiandrosterone; ER, estrogen receptor; MIN, mammary intraepithelial neoplasia; MTD, maximum tolerated dose; PIN, prostate intraepithelial neoplasia; Tag, SV40 large T-antigen; 9-*cis*-RA, 9-*cis*-retinoic acid; 4-HPR, *N*-(4-hydroxyphenyl)retinamide; BrdUrd, bromodeoxyuridine.

In this study, we have used C3(1)/SV40 Tag transgenic mice to examine the potential effects of other chemopreventive agents on this hormone-independent model for mammary cancer. DHEA, which is the primary precursor steroid to both androgens and estrogens in primates but not rodents, has been shown previously by our group as well as others to be highly effective in preventing mammary tumorigenesis in the MNU-induced rat mammary cancer model (17). We also studied the effects of DFMO, which specifically inactivates ornithine decarboxylase (18). This agent has proven to be highly effective in preventing both colon and skin carcinogenesis (19–21), although the initial studies in mammary cancer appear less striking (22).

In addition to using these transgenic mice to screen agents that might be effective against hormone-independent mammary cancer, we used male C3(1)/SV40 Tag transgenic mice to determine the effects of several potential chemopreventive agents, including DFMO, DHEA, 9-*cis*-RA, tocopherol acetate, and selenomethionine, on the development of prostate lesions. We had previously shown that DHEA and 9-*cis*-RA were effective in a rat model of prostate cancer (23), whereas there is some evidence from small clinical trials suggesting efficacy for tocopherol acetate and selenium (selenized yeast) against human prostate cancer (24, 25). The prostates of male C3(1)/SV40 Tag transgenic mice develop both preinvasive and invasive lesions that are histologically similar to those observed in human prostate cancer (26). Because the incidence (100%) and multiplicity of preinvasive lesions are much higher than the incidence of prostate cancer (40%), which takes significantly longer to develop in these mice (26), initial investigations were performed to assess the effects of chemopreventive agents on the incidence and multiplicity of preinvasive prostate lesions.

We demonstrate in this study that DHEA and DFMO significantly inhibit mammary tumor incidence, multiplicity, and tumor burden but that the effect appears to occur after the formation of preinvasive lesions. The compounds tested similarly had no significant effect on inhibition of the development of preinvasive prostate lesions.

MATERIALS AND METHODS

Generation of C3(1)/Tag Transgenic Mice and Administration of Chemopreventive Agents. Transgenic female and male mice heterozygous for the C3(1)/SV40Tag transgene in the FVB/N background and the method for genotyping these mice have been described previously (6). Seven-week-old C3(1)/SV40 Tag mice were randomized into groups of approximately equal weight composed of 21–24 animals/group. Animals were fed Teklad 4% Mouse/Rat Chow as the basal diet or the basal diet supplemented with experimental agents in the feed beginning at 7 weeks of age. Only virgin animals were used for these studies.

Experimental group diets were supplemented with DHEA, DFMO, or 4-HPR. To determine the appropriate dosage of these compounds in the FVB/N strain of mice, toxicity studies were performed in normal FVB/N mice to determine the MTD of each compound. Based on the toxicity assays, C3(1)/SV40 Tag mice received either 0.4 or 0.8 MTD of the chemopreventive compounds.

Mice were weighed twice weekly and palpated weekly for the development of mammary gland tumors beginning at 11 weeks of age. Tumor location and number were measured weekly, and tumor weights were determined for all tumors from all animals at time of sacrifice at 24 weeks of age. Serum samples were obtained at time of sacrifice.

To determine histological changes occurring during the course of treatment, additional groups of mice (6–7 mice/group) were given DFMO or DHEA as described above but sacrificed after 2 or 8 weeks of treatment. Mammary gland tissues and tumors were dissected after euthanasia by CO_2 asphyxiation at the indicated ages. For cell proliferation analyses using BrdUrd, mice were injected with 100 mg/kg BrdUrd i.p. 1 h before sacrifice.

Male mice were obtained from National Cancer Institute Frederick at approximately 4 weeks of age. After 2 weeks of quarantine, mice were randomized into different groups (14–15 mice/group) that received either Teklad diet only (control) or Teklad diet supplemented with 4-HPR (782 ppm), DFMO (8000 ppm), or tocopherol acetate (7500 ppm). Animals remained on the diet until 22 weeks of age. At this time, mice were sacrificed and processed for histopathological analyses. In the case of treatment with selenomethionine, animals were placed on a defined diet, AIN 76, which had very low levels of selenium (0.2 ppm). Control animals were given this diet, whereas the treatment groups were given the same diet supplemented with 10 ppm selenomethionine. 9-*cis*-RA was administered by gavage in corn oil at a dose of 30 mg/kg body weight 5 times/week from 6 weeks of age until 22 weeks of age. Control mice received corn oil alone.

All manipulations of mice were conducted in accordance with the guidelines and procedures outlined in the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, 1985).

Histopathology and Immunohistochemistry. Mammary gland tissues were immediately fixed in 4% paraformaldehyde, 10% phosphate-buffered formalin, or 70% ethanol. Samples were embedded in paraffin, sectioned at a thickness of 4 μm, and stained with H&E for histopathological examination. Histopathological lesions were quantitated by averaging the number of lesions in sections from one axillary and one inguinal mammary gland from each mouse. Glands from the same locations in all mice were used for these studies. Histopathological criteria for diagnosing mammary lesions were as described in the consensus report of the Annapolis Workshop on the mammary pathology of genetically engineered mice (27). Criteria for lesions of the prostate were those described by Shibata (26). The incidence and multiplicity of low-grade and high-grade PIN in the ventral and dorsolateral prostate were determined as described previously.

For immunohistochemical analysis, sections were immersed in distilled water and heated by microwave for antigen retrieval, and the avidin-biotin complex method was performed (Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA). Anti-SV40 Tag mouse monoclonal antibody (PAB 101; PharMingen, San Diego, CA) was used at a dilution of 1:50. Anti-BrdUrd mouse monoclonal antibody Bu20a (DAKO, Carpinteria, CA) was used at a dilution of 1:2000. To evaluate cell proliferation levels, the number of BrdUrd-positive cells was counted in 1000 mammary ductal cells using an ocular micrometer disc (Fisher Scientific, Pittsburgh, PA), and the results were expressed as a percentage (the number of positive cells/100 ductal cells).

Measurement of Apoptosis. Four-μm sections of paraffin-embedded mammary gland tissues were assayed by the terminal deoxynucleotidyl transferase-mediated nick end labeling method as described by the manufacturer (ApopTag; Intergen, Gaithersburg, MD). To quantitate the level of apoptosis, the number of apoptotic cells/1000 cells of a particular type of lesion was counted and expressed as a percentage.

Measurement of Serum Estradiol Levels. Serum estradiol levels were measured by RIA using the Diagnostic Products Corp. kit (Diagnostic Products Corp., Los Angeles, CA) according to the manufacturer's instructions.

RESULTS

Preliminary Toxicity Experiments to Determine MTDs for Chemopreventive Agents

Female Mice. Preliminary toxicity studies for DHEA and DFMO were performed in wild-type female FVB/N mice. DHEA was tested at doses from 250 to 5000 mg/kg. DFMO was tested at doses from 1000 to 10,000 mg/kg. None of the doses resulted in mortality, and the highest dose had no significant effect on body weight.

Male Mice. Preliminary toxicity studies for most of the proposed agents were performed in wild-type male FVB/N mice. The doses chosen for final examination were shown to be nontoxic in the preliminary studies. This was confirmed in the final study because none of the doses used in the final chemoprevention assay significantly affected body weight gain in the treated mice. The doses of vitamin E, selenomethionine, and 9-*cis*-RA were based on published toxicity studies in other strains of mice. These doses were expected to be within a factor of two of the MTDs, based on prior studies that had

showed preventive efficacy for selenomethionine and 9-*cis*-RA in other mouse models (28).

Effects of Chemopreventive Agents on Mammary and Prostate Tumor Growth

To determine the effects of DHEA, DMFO, and 4-HPR on mammary tumor progression, mice were supplemented with these agents in their feed beginning at 7 weeks of age, at which point mammary ductal outgrowth has reached an advanced stage, but MIN lesions (similar to ductal carcinoma *in situ*) are rare in the C3(1)/SV40 Tag transgenic mice (12).

No gross differences in mammary tumor growth were observed in mice that received 4-HPR compared with control mice (data not shown). However, mice that received DHEA or DFMO demonstrated a significant inhibition of tumor incidence, average time to onset, and multiplicity (Figs. 1 and 2) and tumor weight (Table 1). The incidence of grossly palpable tumors arising in mice was reduced by about 20% for groups receiving both high and low doses of these compounds (Figs. 1*A* and 2*A*). This is quite significant, given the fact that essentially 100% of untreated mice develop multiple tumors through the expression of the highly transforming SV40 Tag by this age. Both DHEA and DFMO also significantly reduced tumor multiplicity (Figs. 1*B* and 2*B*) and tumor weights (Table 1 and Fig. 3) by approximately 70%. Regardless of whether or not mice received compounds, the distribution of tumors among the 10 mammary glands

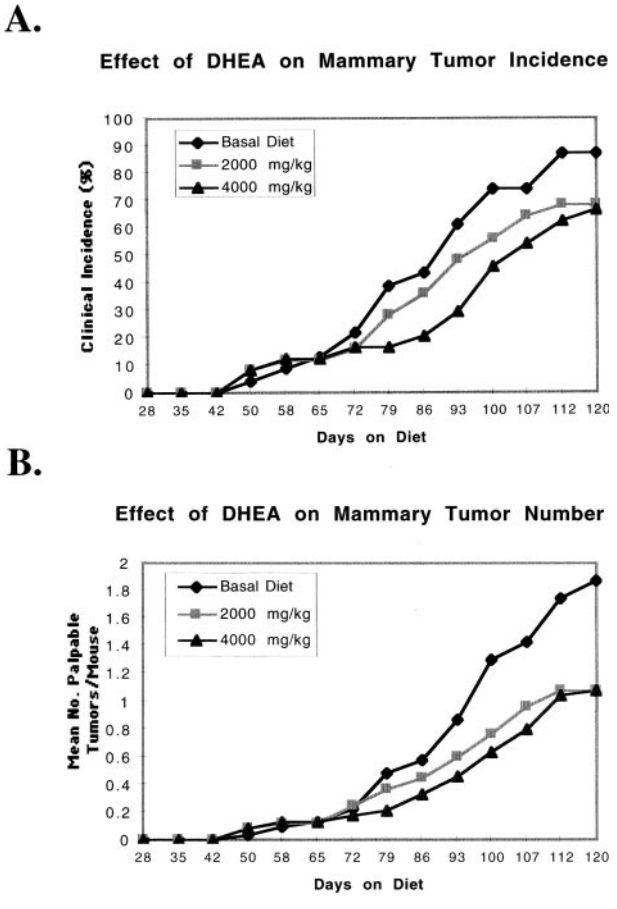

Fig. 1. Inhibition of mammary tumor incidence and multiplicity by DHEA. C3(1)/Tag transgenic mice received either vehicle or 2000 or 4000 mg/kg DHEA beginning at 7 weeks of age. Tumor incidence and multiplicity were recorded weekly. *A*, a dose-dependent inhibitory effect on tumor incidence was observed with a maximum reduction of 20% after 120 days of treatment. *B*, tumor multiplicity was reduced by approximately 50% in the same group of mice.

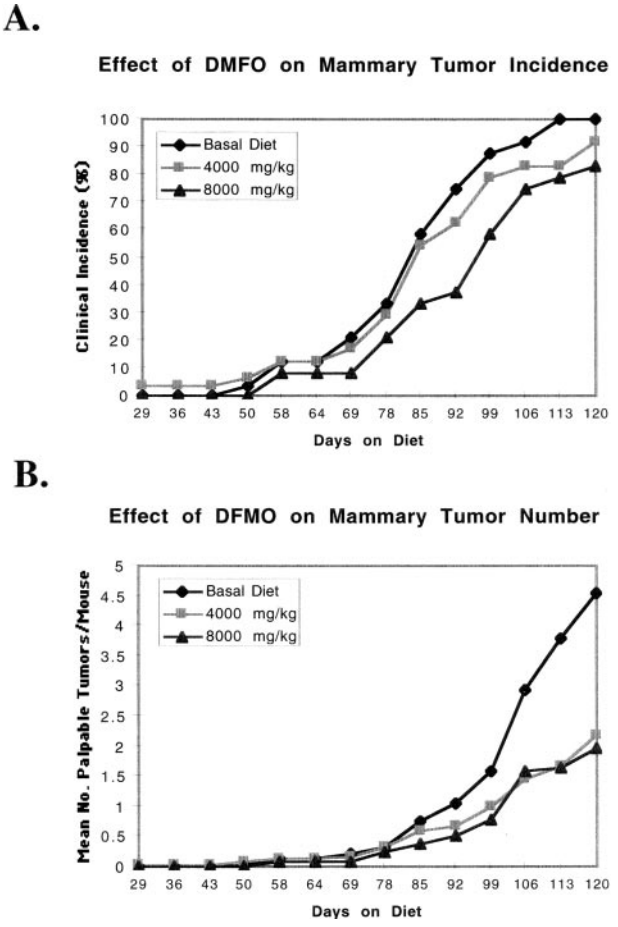

Fig. 2. Inhibition of mammary tumor incidence and multiplicity by DFMO. C3(1)/Tag transgenic mice received either vehicle or 4000 or 8000 mg/kg DHEA beginning at 7 weeks of age. Tumor incidence and multiplicity were recorded weekly. *A*, a dose-dependent inhibitory effect on tumor incidence was observed with a maximum reduction of 20% after 120 days of treatment. *B*, tumor multiplicity was reduced by approximately 50% in the same group of mice.

Table 1 *Dose-response effect of DHEA and DFMO on average mammary tumor weights in C3(1)/Tag transgenic mice*

At the highest dose, DHEA reduced the average tumor weight by 70%, whereas DFMO reduced the average tumor weight by 65%. At similar doses, no changes in body weight were observed for wild-type FVB/N mice. *N*, number of animals, SD, standard deviation.

Treatment	Average tumor weight/animal (g)	*N*	SD	*P*
Experiment A				
Basal diet	3.271	21	3.28	
DFMO				
4000 mg/kg/day	2.560	24	4.10	0.5282
8000 mg/kg/day	0.987	22	1.15	0.0037[a]
Experiment B				
Basal diet	2.788	19	3.06	
DHEA				
2000 mg/kg/day	1.492	24	3.28	0.1919
4000 mg/kg/day	0.981	24	0.96	0.009[a]

[a] Significant difference.

in mice was generally the same. Tumors tended to arise first in the axillary mammary glands (left or right axillary glands 1 and 2), followed by the appearance of tumors in the inguinal glands (left or right inguinal gland 5; Fig. 3). The distribution of tumor growth correlates with the expression of the transgene (6).

Five compounds, including 9-*cis*-RA, selenomethionine, DHEA, DFMO, and α-tocopherol, were tested in male mice to determine their potential effects on prostate lesion development in this transgenic model. Because the development of invasive carcinomas occurs after

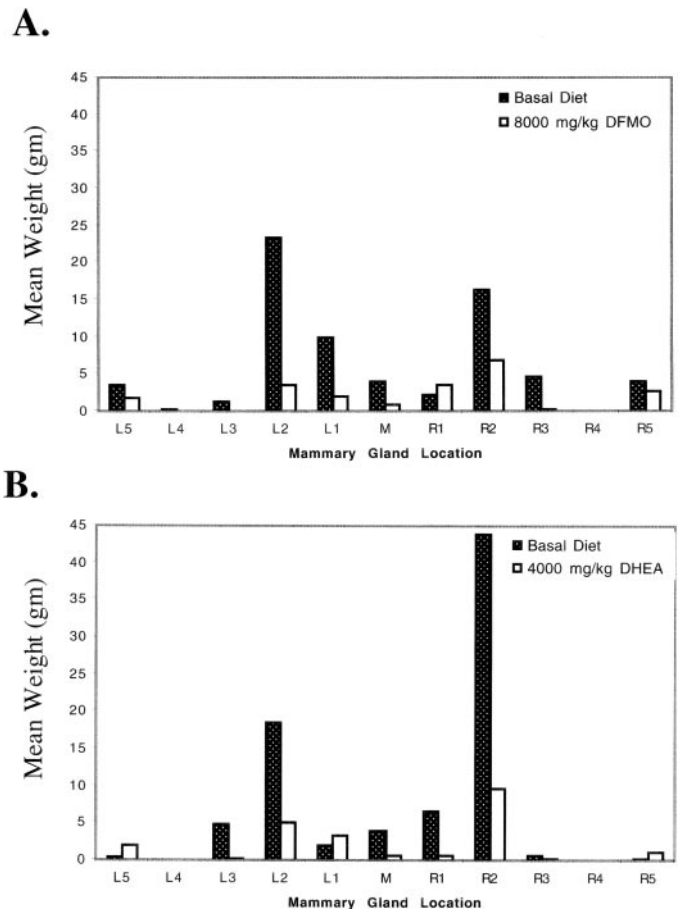

Fig. 3. Distribution and weights of mammary tumors by location. Control mice are compared with C3(1)/Tag mice treated with (*A*) DHEA and (*B*) DFMO. Tumors were dissected individually and weighed at the time of sacrifice (after 120 days of treatment beginning at 7 weeks of age). Tumor location was recorded according to the anatomical location of the mammary glands. *R*, right; *L*, left. The five mammary glands on each half of the animal were designated 1–5, with 1 being the most caudal and 5 being the most distal. Tumors preferentially occurred in axillary glands 1 and 2. Treatment with DHEA or DFMO did not alter relative tumor distribution but significantly reduced tumor weights.

7 months of age in about 40% of the animals, we focused on whether these agents were capable of inhibiting the formation of preinvasive PIN lesions that occur in 100% of the transgenic mice by 5 months of age (26). Three of the agents were administered in the diet. 9-*cis*-RA was administered by gavage because we have observed previously that mice have an apparent taste aversion to this compound even at relatively low doses. The other two agents that we examined were the antioxidant α-tocopherol, which was administered in the standard diet, and selenomethionine, which was administered in a diet with a known low concentration of selenium. None of the agents demonstrated significant effects on the incidence or multiplicity of PIN lesions. We also examined the effect of DFMO, which we demonstrated is effective in inhibiting hormone-independent mammary tumorigenesis in the C3(1)/SV40 Tag model. No significant effect on the incidence or multiplicity of preinvasive prostate lesions even at the relatively high dose of DFMO that we used (data not shown).

Effects of Chemopreventive Agents on Histopathology of Mammary and Prostate Lesion Progression

Although DHEA and DMFO significantly inhibited tumor development, the pathology of the mammary lesions that arose was similar at all stages to that observed in the control animals. The invasive tumors were generally classified as adenocarcinomas, as has been described previously for this transgenic model (11, 12). Thus, these compounds inhibited the development of mammary lesions but did not appear to alter the state of differentiation of the tumors.

To determine at what stage these compounds affected tumor progression, mice were sacrificed 2 or 7 weeks after the initiation of therapy (at 9 weeks or 14 weeks of age), and histopathological analyses were performed to quantitate the number of MIN lesions. The number of MIN lesions was not significantly lower in mice that received DHEA or DMFO. At 14 weeks of age, the average number of MIN lesions was 2.54 ± 2.64 in the control mice ($n = 12$ mammary glands), 2.50 ± 1.64 in the DHEA-treated group ($n = 14$ mammary glands), and 4.32 ± 3.69 in the DFMO-treated group ($n = 14$ mammary glands). These results suggest that DHEA and DFMO do not inhibit MIN development but retard the further progression of tumor growth either during the transition from preinvasive to invasive lesions or after the lesions have progressed to invasive carcinomas.

Male mice received compounds from 7 weeks of age until 20 weeks of age, at which time they were sacrificed. There were no differences in the histopathological characteristics of the PIN lesions between mice that received compound and mice that received vehicle (data not shown). The prostate lesions were identical to those reported previously (26).

Effect of DHEA and DFMO on Cell Proliferation and Apoptosis

To determine whether the reduction in tumor progression observed in the mice treated with DHEA and DFMO was due to an alteration in the rate of either cell proliferation or apoptosis, BrdUrd labeling and terminal deoxynucleotidyl transferase-mediated nick end labeling staining were performed on mammary lesions after 2 weeks of treatment. There were no significant differences in the rates of cell proliferation or apoptosis in early MIN lesions between C3(1)/Tag mice treated with either DHEA or DFMO and untreated animals (Fig. 4). These results further suggest that the inhibitory effects of these compounds occur at a later stage of tumor progression.

Effect of Chemopreventive Agents on Transgene Expression and Serum Estradiol Levels

Because the level of transgene expression will determine the dynamics of tumor development and formation, the levels of Tag expression were examined in mammary tissues from control and treated mice. No difference in Tag levels was observed in the mammary epithelium between any groups, as assessed by a scoring system using immunohistochemical staining (data not shown). This is consistent with our previous work (12).

Animals that received either DHEA or DMFO had significant elevations in serum estradiol levels as measured by RIA. Serum levels of estradiol were measured in mice 2 weeks after commencing treatment with DHEA. The serum estradiol level was 23.4 pg/ml in mice receiving 2000 mg/kg DHEA, 39.8 pg/ml in mice receiving 4000 mg/kg DHEA, and 9.25 pg/ml in untreated control mice. Although estradiol levels are higher in treated mice, elevation of estradiol levels does not affect C3(1)/SV40 Tag transgene expression (13). However, sustained high doses of estradiol do appear to promote mammary tumor development in C3(1)/Tag transgenic mice (13).

DISCUSSION

The C3(1)/SV40 Tag model is unique in that the same oncogene is expressed in both the mammary and prostate epithelium, leading to the development of cancer in both organs with many histological features of human breast and prostate cancer (6, 11, 12, 26). Although

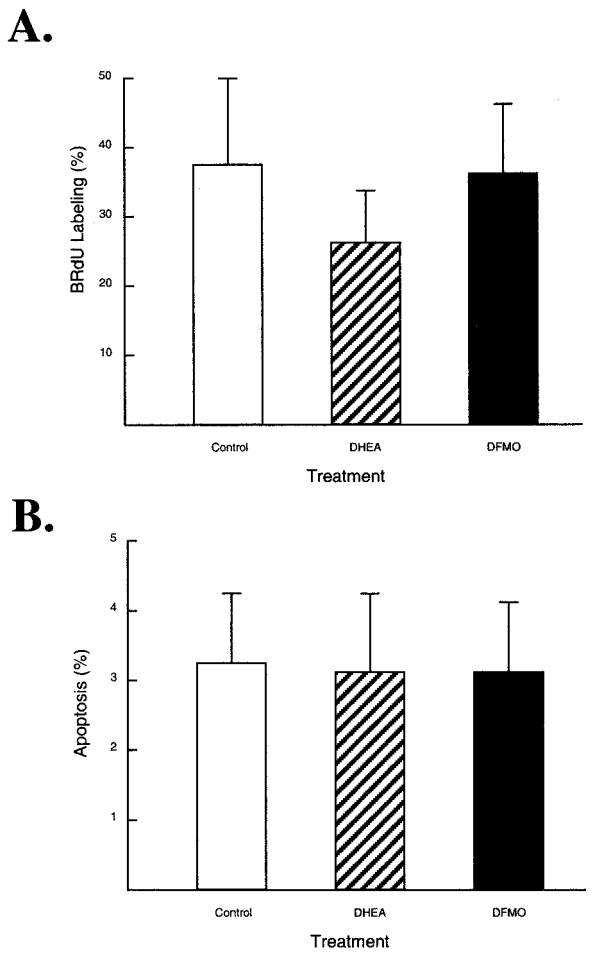

Fig. 4. Effects of DHEA and DFMO on rates of proliferation and apoptosis in preinvasive mammary lesions. Mice were treated with vehicle, DHEA, or DFMO beginning at 7 weeks of age and sacrificed at 12 weeks of age, a time when numerous MIN lesions are present, but few invasive carcinomas have developed. *A*, rates of proliferation were measured by determining the percentage of cells in MIN lesions that stained positively for BrdUrd. There was no statistically significant effect on BrdUrd incorporation by DHEA or DFMO. *B*, levels of apoptosis were measured by determining the percentage of cells within MIN lesions that stained positively using the ApopTag method. No differences in levels of apoptosis were observed in C3(1)/Tag mice that received vehicle compared with mice that received DHEA or DFMO.

the rat C3(1) gene is most highly expressed in the rat ventral prostate, the major androgen-responsive element contained in the first intron was not included in the transgenic construct, which likely accounts for the high level of transgene expression in the mammary epithelium.

In our previous work, we have extensively characterized the natural history and histopathology of tumor development in this model (6, 11, 12, 26), the response to estrogen signaling (13), and genetic and genomic alterations that occur during tumor progression (14, 15, 26, 29). In contrast to the profound chemopreventive effects observed in the hormone-responsive rat mammary tumor system, we have shown that neither ovariectomy nor tamoxifen treatment profoundly alters tumor development (incidence or multiplicity) in the C3(1)/SV40 Tag model (13). In addition, we have recently shown that the expression of ER-α is greatly diminished during tumor progression in this model, further demonstrating that the mammary tumors are estrogen independent (13).

Based on these observations, we wished to determine whether several promising chemopreventive agents would have efficacy in this transgenic model in which hormone-independent mammary cancers develop in female mice, and prostate cancers develop in male mice with the same genetic background. As shown in Fig. 1, we obtained a significant reduction in mammary cancer in animals treated with DHEA. We chose doses based on toxicity studies and estimate that animals received up to 50 times the dose of DHEA routinely used in human studies, based on consumption in their diet. Because DHEA is not a normal steroid hormone metabolite in rodents, it is difficult to make a correlation between human and mouse therapeutic levels. A number of investigators, including ourselves, have observed significant effects of DHEA on body weight gain in A/J mice with dietary supplementation at doses as low as 500 mg/kg body weight. However, our toxicology results in FVB/N mice using 2500–5000 mg/kg DHEA in the diet showed no alteration in weight gain, which strongly supports our findings that DHEA clearly has chemopreventive effects on mammary tumor development in this model unrelated to weight effects. The reason for these large strain differences in response to DHEA was unexpected, and although we did not obtain serum levels of DHEA, we did observe significant increases in serum estradiol, indicating that we had achieved absorption and a significant physiological effect. Our previous studies in rats with this compound had shown that even at extremely low doses, DHEA profoundly decreased the development of MNU-induced tumors in this hormone-responsive rat mammary tumor model (17). Although levels of the steroids DHEA and its conjugate, DHEA sulfate, are very high in primates, they are not found at significant levels in rodents. DHEA is the primary precursor of estrogens and androgens, and as such, DHEA supplementation in rats greatly increases serum levels of both androgens and estrogens (17). We similarly demonstrate in this study that DHEA increased the levels of estrogen in the serum of treated mice by 2–3-fold. Despite this modest increase in serum estradiol levels by DHEA, tumorigenesis was nonetheless inhibited by DHEA. Because we have previously shown that supplemental estradiol at much higher concentrations than that induced by DHEA leads to a significant increase in MIN formation and mammary tumorigenesis in the C3(1)/Tag model (13), the chemoprotective effect of DHEA appears to be substantially stronger than the potential tumor-promoting effect of the increased estradiol induced by DHEA. The fact that we did not observe a significant increase in MIN lesions in DHEA-treated mice despite the elevated serum estradiol levels suggests that DHEA might also have a role in inhibiting MIN lesion formation. However, it seems more likely that the DHEA-induced increase in estradiol levels may be too low to stimulate MIN formation. Because the number of MIN lesions at 14 weeks was similar in the control and DHEA-treated groups but the number of tumors that emerged in the DHEA-treated mice was substantially less than that for the controls, we conclude that DHEA inhibits the progression of MIN lesions to invasive carcinomas or the further growth of early invasive carcinomas.

The exact mechanism by which DHEA exerts its chemopreventive effects in the mammary gland is not known, although it has been postulated that DHEA may increase differentiation of the mammary gland. DHEA has similarly been found to be effective in a rat model of prostate cancer (30), despite the fact that it may have a significant androgenic effect by increasing the levels of testosterone and dihydrotestosterone (30). DHEA is also known to activate peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ, which might induce differentiation and inhibit tumorigenesis (31).

Our results indicate that DHEA is effective in a hormonally nonresponsive model of mammary cancer, despite causing elevated estradiol levels. This result may bode well for the use of such agents, which may act in part by differentiating the mammary epithelium and thereby inhibiting the formation of both hormone-responsive and hormone-independent breast cancer.

The second compound that we examined, DFMO, is a relatively specific noncompetitive inhibitor of ornithine decarboxylase. This agent has previously been shown to be highly effective in a number of

animal models of cancer including colon, skin, and tongue (18–21). In contrast to published studies with DHEA, the data for DFMO in mammary tumor models are more limited, and the published data are not striking (22). Nevertheless, we observed a highly significant effect on tumor incidence, multiplicity, and tumor burden in the animals treated with DFMO. When we examined the effects of DFMO on preinvasive lesions, we observed no difference in the incidence of MIN lesions, similar to our result for DHEA. Therefore, like DHEA, DFMO appears to exert its inhibitory effects after the early stages of tumor (MIN) development. This apparent effect of DFMO during the later stages of mammary tumor progression in the C3(1)/Tag model is consistent with our findings in human papillomavirus-induced (32) and UV-induced (33) skin cancer models, in which DFMO is able to reverse preexisting skin lesions. Administration of DFMO to rats 12 weeks after exposure to a carcinogen is also highly effective in inhibiting colon cancer progression (data not shown). These results strongly suggest that both DHEA and DFMO inhibit tumor progression after MIN formation. This study further demonstrates that the C3(1)/SV40 Tag model can be used to screen for chemopreventive agents useful against hormone-independent mammary tumors.

Although both DHEA and DFMO were able to reduce tumor incidence, multiplicity, and volume, we were not able to demonstrate that these agents reduced rates of cellular proliferation or increased apoptosis in lesions after 2 weeks of treatment. It is possible that a later time point may have demonstrated differences in these parameters or that more sensitive measurements of the growth fractions may have yielded a difference. However, given the fact that the progression from MIN to invasive, palpable lesions is a relatively rare event (at least during the life span of these animals), these assays would not be sensitive enough to identify the differences in the small number of early lesions that are able to progress to advanced tumors. Thus, these agents might retard a relatively rare but critical process in tumor progression.

For studies involving prostate oncogenesis in the C3(1)/Tag mice, we examined the effects of five agents that have been associated with reducing prostate cancer development in previous studies. Four of these agents have demonstrated some efficacy in animal or human studies supporting their potential use as chemopreventive agents for prostate cancer. DHEA and 9-*cis*-RA, a pan-retinoid receptor agonist, have previously been shown to be modestly effective in a rat prostate tumor model (2/3). Both α-tocopherol and selenium have been shown to be partially effective in reducing prostate cancer in human trials with small cohorts (24, 25). We also studied the effect of DFMO on prostate lesion development to compare its effect with that observed for mammary tumorigenesis. None of these agents were effective in reducing the development of PIN lesions in this model. α-Tocopherol and selenomethionine have also not been effective in reducing prostate cancer in a rat prostate model (34).

Although DHEA and DFMO reduced mammary tumorigenesis in the C3(1)/SV40 Tag model, we cannot conclude from our studies that these agents are ineffective against prostate cancer formation in this model. Because we only looked at the effects on preinvasive lesions in the prostate, it remains quite possible that DHEA and DFMO might be efficacious in reducing the formation of invasive prostate carcinomas, as we observed for the mammary tumors. This question will be addressed in future studies.

Whereas it has been generally assumed that chemopreventive agents act at very early stages of cancer development, the results of this study suggest that the inhibition of mammary cancer progression by DHEA and DFMO occurs after the development of preinvasive lesions. Because this is the stage where ER expression appears to be lost in the C3(1)/Tag model (13), these results may have important implications for the prevention of ER-negative breast cancer in humans. The fact that our data as well as that of other studies using DFMO in skin cancer models (32, 33) and cyclooxygenase-2 inhibitors in gastrointestinal cancer models demonstrate an inhibitory effect on tumor progression after the development of early lesions suggests that these chemopreventive agents may act well beyond the earliest stages of tumor initiation. The concept of chemoprevention might therefore be expanded to include prevention of tumor progression.

This work, which demonstrates that the C3(1)/SV40 Tag model responds to two mechanistically unrelated agents, supports the further use of this model for the testing of selected chemopreventive agents. Although the antiestrogens tamoxifen and raloxifene have been shown to be effective chemopreventive agents against the development of hormone-responsive breast cancer (35), the development of chemopreventive agents against hormone-independent tumors is of great clinical importance. Because the mammary tumors that develop beyond the MIN stage in the C3(1)/SV40 Tag model are ER negative and hormone independent, the results of this study as well as those of our previous study demonstrating that 9-*cis*-RA inhibits mammary tumorigenesis in this model (16) may be highly relevant to chemoprevention of hormone-independent human breast cancer. Further analyses using additional parameters including gene expression profiling to assess the response of the transgenic tumors to chemopreventive agents will help address the validity of this model and may help identify additional molecular targets for chemoprevention.

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