Chemoprevention of Prostate Carcinogenesis by α-Difluoromethylornithine in TRAMP Mice

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ABSTRACT

Development of effective chemopreventive agents for human consumption requires conclusive evidence of their efficacy in animal models that have relevance to human diseases. Transgenic adenocarcinoma mouse prostate (TRAMP) is an excellent model of prostate cancer that mimics progressive forms of human disease inasmuch as 100% of males develop histological PIN by 8–12 weeks of age that progress to adenocarcinoma with distant site metastases by 24–28 weeks of age. In these animals, ornithine decarboxylase (ODC) activity (>3-fold) as well as protein expression (>4-fold) was found to be markedly higher in the dorsolateral prostate as compared with the nontransgenic littermates, suggesting their suitability to determine the chemopreventive effect of α-difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor of ODC, against prostate cancer. Using male TRAMP mice, we studied the effect of oral consumption of DFMO on development of prostate carcinogenesis and surrogate end point biomarkers related to prostate cancer progression. In two independent experiments, each consisting of 8 animals on test, the cumulative incidence of prostatic cancer development at 28 weeks of age in 16 untreated TRAMP mice was 100% (16 of 16), whereas 94% (15 of 16) and 69% (11 of 16) of the animals exhibited distant site metastases to lymph nodes and lungs, respectively. Oral consumption of 1% DFMO (w/v) in the drinking water to TRAMP mice from 8 to 28 weeks of age resulted in a significant decrease in (a) weight (59%) and volume (66%) of prostate, (b) genitourinary weight (63%), and (c) ODC enzyme activity (52%) in the dorsolateral prostate. Importantly, in none of the DFMO-fed TRAMP mice were any distant metastases to lymph node and lungs observed. Furthermore, DFMO treatment resulted in the marked reduction in the protein expression of proliferation cell nuclear antigen, ODC, and probasin in the dorsolateral prostate. The protein expression of antimesatases markers, i.e., E-cadherin and α- and β-catenin, was found to be restored in DFMO-fed animals as compared with the non-DFMO-fed mice. These chemopreventive effects of DFMO were further confirmed by immunohistochemical analysis of the dorsolateral prostate. Histological analysis of the dorsolateral prostate of DFMO-fed animals displayed marginal epithelial stratification, a small number of cribriform structures, elongated hyperchromatic epithelial nuclei, and a significant increase in apoptotic index. Non-DFMO-fed animals, on the other hand, displayed extensive epithelial stratification with profound cribriform structures accompanied with marked thickening, remodeling, and hypercellularity of the fibromuscular stroma. In nontransgenic littermates fed with DFMO, no significant alterations in the above parameters were evident. These data demonstrate that ODC represents a promising and rational target for chemoprevention of human prostate cancer and that TRAMP mice are excellent models for screening of novel drugs and chemopreventive regimens for potential human use.

INTRODUCTION

CaP is the most commonly diagnosed solid tumor and the second leading cause of cancer mortality in American men (1). According to an estimate by the American Cancer Society, ~18% of the male population in the United States will develop invasive CaP during their lifetime (1, 2). The development of CaP in humans has been viewed as a multistage process, involving the onset as small latent carcinoma of low histological grade to large metastatic lesion of higher grade (3). Unfortunately, there are limited treatment options available for this disease because chemotherapy and radiation therapy are largely ineffective, and metastatic disease frequently develops even after potentially curative surgery (4–6). Chemoprevention could be an effective approach to reduce CaP incidence (7, 8). Indeed, CaP is an excellent candidate disease for chemoprevention because of its diagnosis in elderly men, and therefore even a modest delay in the neoplastic development achieved through pharmacological or nutritional intervention could result in a substantial reduction in the incidence of this clinically detectable disease (9).

For relevance to human population, chemoprevention studies should be conducted in animal models that mimic progressive forms of human disease and possess surrogate end point biomarkers for rapid screening of drugs (10–12). In recent years, genetically manipulated animal models have emerged as resources for developing strategies against many pathological conditions, including cancer (13–15). Advantages of these genetically manipulated animals for chemoprevention studies include induction of carcinogenesis by discrete genetic changes and the ability to modulate oncogenes or tumor suppressor genes implicated in human cancers (16–18). One strength of transgenic models is that in these animals, cancer arises from normal cells in their natural tissue microenvironment and progress through multiple stages, as does human cancer (19).

For CaP very few genetically manipulated models are available (20, 21). One such model is TRAMP (22). TRAMP mice express a PB-Tag transgene consisting of the minimal −426/+28-bp regulatory element of the rat probasin promoter directing prostate-specific epithelial expression of the SV40 early genes (T/t antigens; Refs. 22 and 23). TRAMP is an excellent model that serves as a general prototype of the pathways, parameters, and molecular mechanisms of multistage prostate tumorigenesis (22). TRAMP males develop spontaneous multistage prostate carcinogenesis that exhibits both histological and molecular features similar to that of human CaP (24). TRAMP males characteristically express the PB-Tag transgene by 8 weeks of age and develop distinct pathology in the epithelium of the dorsolateral prostate by 10 weeks of age. Distant site metastasis can be detected as early as 12 weeks of age in male TRAMP mice, and by 28 weeks of age, 100% of the animals harbor CaP that metastasizes to the lymph nodes and lungs (22, 24).

In the present study, we determined the use of TRAMP mice for...
CaP chemoprevention studies. We used DFMO as a chemopreventive agent to investigate its efficacy for CaP. Our choice of DFMO is based on the fact that it is an irreversible “suicide inhibitor” of the enzyme ODC, and ODC is being increasingly appreciated as a target for CaP chemoprevention because of the following reasons: (a) ODC is shown to play a role during CaP development in the murine prostate carcinogenesis model (25–27), (b) ODC is overexpressed in prostate tissue and prostatic fluid in human subjects with CaP (28), and (c) our studies have shown that ODC enzyme activity and protein expression are elevated in the dorsolateral prostate of TRAMP mice. The results from this study suggested that ODC is a rational target and end point biomarker for CaP development and progression, and TRAMP is an excellent model for screening for novel drugs and chemopreventive agents against CaP.

MATERIALS AND METHODS

Animals. The male and female heterozygous TRAMP mice, line PB-Tag 8247, were developed in the laboratory of one of us (N. M. G.) at Baylor College of Medicine (Houston, Texas) and were bred and maintained at Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) internationally accredited Animal Resource Facility of Case Western Reserve University. All of the experiments were conducted using the highest standards for animal care in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. Transgenic males for these studies were routinely obtained as [TRAMP × C57BL/6]F1 or as [TRAMP × C57BL/6]F2 offspring. Identity of transgenic mice was established by the PCR-based DNA screening as described earlier (29).

ODC Enzyme Activity. Male TRAMP mice (8, 16, and 24 weeks old) and their nontransgenic littermates of the same age were used for initial studies. The animals had access to food and water ad libitum, and in each group, six animals were used. The animals from both transgenic and nontransgenic groups were sacrificed by cervical dislocation, and dorsolateral prostate was removed. The tissue was homogenized at 4°C in a glass-to-glass homogenizer in 10 volumes of ODC buffer [50 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM EDTA, 1 mM pyridoxal-5-phosphate, 1 mM 2-mercapto-ethanol and 0.1% Tween-80]. The homogenate was centrifuged at 14,000 × g at 4°C, and the supernatant was used for enzyme determination. ODC activity was determined by measuring the release of 14CO2 from L-[1-14C]ornithine hydrochloride, as described earlier (28). Briefly, 100 μl of the supernatant were added to 0.25 ml of the assay mixture [35 mM sodium phosphate (pH 7.2), 0.2 mM pyridoxal phosphate, 4 mM DTT, 1 mM EDTA, 0.4 mM L-t-ornithine containing 0.5 μCi of DL-L-[1-14C]ornithine hydrochloride] in a 15-ml corex centrifuge tube equipped with rubber stoppers and central well vessels containing 0.2 ml of ethanolamine and methoxyethanol in a 2:1 (v/v) ratio. After incubation at 37°C for 60 min, the reaction was terminated by the addition of 0.5 ml of 2 M citric acid using a 21-gauge needle/syringe. The incubation was continued for 1 h. Finally, the central well containing the ethanolamine/methoxyethanol mixture to which 14CO2 has been trapped was transferred to a vial containing 10 ml of toluene-based scintillation fluid and 2 ml of ethanol. The radioactivity was measured in a Beckman LS 6000 SC liquid scintillation counter.

Study Design for DFMO Chemoprevention. The effect of 1% DFMO (w/v) consumption on prostate carcinogenesis in TRAMP mice was studied in two independent experiments. Throughout the experiment all of the animals had ad libitum access to laboratory chow. In each experiment, 16 male TRAMP mice of 8 weeks of age were divided in two groups. The experimental group was provided with 1% DFMO (ILEX Oncology, San Antonio, TX) in drinking water (w/v) for 20 weeks, whereas the control group was fed with 1% DFMO (w/v) for 14 weeks and later returned to regular drinking water for 6 weeks, was sacrificed, and was studied for prostate tumorigenesis as described earlier.

Immunoblotting. The dorsolateral prostate was removed from the transgenic and nontransgenic littermates, homogenized in lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na3VO4, 0.5% NP40, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin (pH 7.4)] at 4°C to prepare cell lysates. Appropriate amount of protein (25–50 μg) was resolved over 8–14% Tris-Glycine polyacrylamide gel and then transferred onto the nitrocellulose membrane. The blots were blocked using 5% nonfat dry milk and probed using appropriate primary antibody of ODC (Neomarkers Inc., Union City, CA), mPB-1 (from N. M. G.’s laboratory), E-cadherin, PCNA, and α- and β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer overnight at 4°C. The membrane was then incubated with antimouse or antirabbit secondary antibody HRP conjugate (Amersham Life Sciences Inc., Arlington Heights, IL) followed by detection using the enhanced chemiluminescence kit (Amer- sham Life Sciences Inc., Arlington Heights, IL). Equal loading of protein was confirmed by stripping the membrane and reprobing it with β-actin primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and appropriate secondary HRP conjugate.

Tissue Processing and Histopathology. The dorsolateral prostate was excised and fixed overnight in (10%) zinc-buffered formalin and then transferred to 70% ethanol. Sections (4 μm) were cut from paraffin-embedded tissue and mounted on slides. The sections were stained with H&E as described previously (24). Histological sections were reviewed by light microscopy for the presence of CaP and classified as PIN (epithelial stratification with occasional mitotic figures or cribriform pattern), well- (multiple epithelial mitotic figures and apoptotic bodies, invasive glands with stromal hypercellularity), moderately (many acini completely filled with tumor yet still forming some glandular structures), or poorly (sheets of malignant cells with little or no glandular formation) differentiated CaP; or atrophic glands only (no identifiable tumor deposits).

Immunohistochemical Analysis. Sections (4 μm) were cut from paraffin-embedded tissues. Immunostaining was performed using antibody for mPB-1, PCNA, E-cadherin, and α- and β-catenin with appropriate dilutions and was replaced with either normal host serum or block for negative controls, followed by counterstaining with either a weak hematoxylin stain or methyl green as described previously (22, 24). The stained slides were visualized on a Zeiss-Axioshot DM HT microscope (Zeiss-Axioshot, Germany). Images were captured with an attached camera linked to a computer.

Metastasis Examination. The India ink method was used to examine cancer metastasis to lungs. For this, the animals were sacrificed and the respiratory system was excised. India ink was injected through the trachea into the lung and filled with a 5-ml syringe using a fine tip until the lungs were completely filled with ink. The trachea was then tied with a thread. The ink absorbs in whole tissue, with the exception of places where the metastasis had occurred. Thus, the metastatic tissues were visible as unstained spots in lungs, which were stained blue.

Scoring of Apoptotic Cells. Apoptotic cells were evaluated by the morphological examination of H&E-stained sections under light microscopy. Scoring was done under the microscope using the Optimas 6 software program (Optimas Corp., Bothell, WA). Apoptotic index (%) was calculated by dividing the number of apoptotic cells by the total number of cells counted per cross-section of a sample of the prostate.

Densiometry and Statistical Analysis. Densitometric measurements of the bands in immunoblot analysis were performed using the Scion Image computer program (Scion Corp., Frederick, MD). The data are expressed as mean ± SE. The significance between the control and experimental groups was obtained by using the Student’s t test, and a P ≤ 0.05 was taken as significant in all of the experiments.

RESULTS

ODC Enzyme Activity and Protein Expression in TRAMP Mice. ODC, the rate-limiting enzyme in polyamine biosynthesis pathway, is associated with growth and development of neoplastic regrowth, in another set of experiment, 12 male TRAMP mice of 8 weeks of age were divided into two groups. The control group was fed with 1% DFMO (w/v) for 20 weeks, whereas the experimental group was fed with 1% DFMO (w/v) for 14 weeks and later returned to regular drinking water for 6 weeks, was sacrificed, and was studied for prostate tumorigenesis as described earlier.

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tissue and is found to be up-regulated in almost all epithelial cancers, including CaP (33–35). In this experiment, we first compared the levels of ODC enzyme activity and protein expression in dorsolateral prostate of TRAMP mice and their nontransgenic littermates at 8 weeks of age. As shown in Fig. 1A, basal levels of ODC enzyme activity were similar in nontransgenic and TRAMP mice. ODC activity in 16- and 24-week-old TRAMP mice was 2.5- and 3.3-fold higher than in age-matched nontransgenic littermates. The ODC protein expression was significantly higher in 16- and 24-week-old TRAMP mice (4.4-fold and 5.6-fold, respectively) compared with the nontransgenic littermates (Fig. 1B). This observation offers the unique opportunity to use the TRAMP model for DFMO chemoprevention studies.

Effect of DFMO Consumption on Prostate Tumorigenesis in TRAMP Mice. DFMO feeding for 20 weeks did not significantly affect the body weight gain profile of nontransgenic littermates compared with their corresponding controls. In contrast, TRAMP mice fed with DFMO were 8 g lighter, 28 ± 3 versus 36 ± 4, than the non-DFMO-fed TRAMP mice. The difference in the body weight appeared to be attributable to the hyperproliferation of the GU apparatus and local abdomen region in the non-DFMO-fed TRAMP mice (Fig. 2).

To investigate the effects of DFMO consumption on CaP in TRAMP mice, in two independent experiments, DFMO was fed for 20 weeks starting at 8 weeks of age to TRAMP mice and their nontransgenic littermates. As summarized in Table 1, in the first experiment, eight of eight TRAMP mice in the non-DFMO-fed group developed severe CaP with remarkable local invasiveness, and distant site metastases to lymph (seven of eight) and lungs (six of eight) were also observed. In contrast, none of the eight DFMO-fed TRAMP mice on the test developed metastases to lymph nodes or lungs. However, in all of the animals, mild local invasiveness was observed in the seminal vesicles and local prostate region. Similarly, in the repeat experiment, all eight mice in the non-DFMO-fed group developed fully malignant invasive CaP with metastases to lymph (eight of eight) and lungs (five of eight). At the termination of the experiment, at 28 weeks in the DFMO-fed group, none of the eight mice exhibited any metastases to lymph nodes and lungs.

We next investigated the effect of DFMO consumption on the GU apparatus in TRAMP mice. As observed visibly, DFMO feeding resulted in almost none to complete absence of hyperplasia in the GU region, especially of the seminal vesicles. The most striking features of DFMO consumption were evident by a significant decrease in prostate weight (59%) and volume (66%) as compared with the non-DFMO-fed TRAMP mice. No significant changes were observed in the DFMO-fed nontransgenic littermates as compared with their corresponding control (Table 1).

Effect of DFMO Consumption on Histological Features in Prostate of TRAMP Mice. As shown in Fig. 3A, a typical dorsolateral prostate from a control, nontransgenic littermate is composed of acini with abundant eosinophilic intralumenal secretions. The acini are lined by a layer of well-organized columnar secretory epithelium possessing round and inconspicuous nuclei. A single layer of thin, flat basal epithelial cells with elongated nuclei typically surrounds the columnar epithelium, and a fibromuscular stroma containing three to five layers of smooth muscle surrounds the entire prostate. In contrast, the prostate of the DFMO-fed TRAMP mice exhibited a more fibromuscular stroma and a reduced number of acini. The acini were smaller and more disorganized, and the basal epithelial cells were more evident. These changes were more pronounced in the DFMO-fed TRAMP mice than in the non-DFMO-fed TRAMP mice. The acini in the non-DFMO-fed TRAMP mice were more similar to those in the control, nontransgenic littermate. The acini in the DFMO-fed TRAMP mice were more similar to those in the non-transgenic control.
four cell layers of stratified smooth muscle surrounds the acinus. The dorsolateral prostate of the nontransgenic littermate supplemented with DFMO for 20 weeks was entirely similar to that of the nontransgenic water-fed group (Fig. 3A).

A detailed histopathological investigation of neoplastic progression of the dorsolateral prostate of non-DFMO-fed TRAMP mice at 28 weeks of age displayed poorly differentiated PIN characterized by profound cribriform structures and numerous apoptotic bodies, invasive glands accompanied by marked thickening, remodeling, and hypercellularity of the fibromuscular stroma (Fig. 3A). In sharp contrast, the experimental group of DFMO-fed TRAMP mice exhibited well-differentiated epithelial stratification with a marked reduction in cribriform structures, thickening, and remodeling of the fibromuscular stroma underlying the abnormal prostatic epithelium along with little or no glandular formation (Fig. 3A). Compared with the non-DFMO fed TRAMP mouse, the prostate of DFMO-fed mouse exhibits acini with many epithelial nuclei, which are elongated and hyperchromatic, with clumping of the chromatin and an increased nuclear:cytoplasmic ratio. DFMO feeding also resulted in a significant increase in the number of apoptotic cells, which occurred sporadically and are darkly stained (Fig. 3B). A high apoptotic index was observed in the DFMO-fed group, compared with non-DFMO-fed TRAMP mice (Fig. 3C).

**Effect of DFMO Consumption on ODC Activity and Protein Expression in TRAMP Mice.** TRAMP males exhibit high basal levels of ODC enzyme activity and protein expression in the dorsolateral prostate, which is associated with cell invasion and regarded as a marker of cell proliferation (16, 25, 28, 30). As shown in Fig. 4A, DFMO feeding for 20 weeks to TRAMP mice (ODC activity, 218.9 ± 15.3 pmol/h/mg protein) resulted in a significant decrease ($P < 0.001$) in ODC enzyme activity in the dorsolateral prostate as compared with the non-DFMO-fed TRAMP mice (ODC activity, 454.4 ± 31.8 pmol/h/mg protein). Importantly, TRAMP mice fed with DFMO for 20 weeks also showed a significant reduction in ODC protein expression in the dorsolateral prostate as compared with the non-DFMO-fed TRAMP group (Fig. 4B). The densitometric analysis of the bands (normalized for β-actin) showed a significant decrease ($P < 0.001$) in ODC protein expression (54%) in DFMO-fed TRAMP mice compared with the non-DFMO-fed TRAMP mice (Fig. 4C). DFMO supplementation to the nontransgenic littersmates did not cause any significant alterations in the ODC activity and protein expression in the dorsolateral prostate (Fig. 4).

**Effect of DFMO Consumption on Proliferation Marker(s) in TRAMP Mice.** TRAMP mice reproducibly express the PB-Tag transgene in a male-specific and developmentally regulated fashion. Probasin (PB) is an androgen responsive gene expressed specifically in prostate epithelial cells and has been shown to be up-regulated in CaP (34–36). Therefore, we next determined the effect of DFMO consumption on the protein expression of probasin in the dorsolateral prostate of these mice. TRAMP mice fed with DFMO for 20 weeks exhibited significant inhibition in probasin protein expression in the dorsolateral prostate (Fig. 5A). These results were further confirmed by comparing the relative densities of the bands where 49% reduction ($P < 0.001$) was observed in the DFMO-fed TRAMP mice as compared with the non-DFMO-fed TRAMP animals (Fig. 5B). The immunohistochemical staining of the dorsolateral prostate in DFMO-fed TRAMP mice exhibited a significant decrease in probasin protein expression as compared with the non-DFMO-fed TRAMP group (Fig. 6). However, in the nontransgenic littersmates, DFMO supplementation was not found to result in noticeable alterations in the probasin expression compared with their corresponding control (Figs. 5 and 6).

Because DFMO is a known antiproliferative agent (37, 38), we investigated the effect of DFMO chemoprevention on the ubiquitous and molecular marker for increased proliferation, PCNA. PCNA serves as a requisite auxiliary protein for DNA polymerase δ-driven DNA synthesis and is cell cycle regulated (39). DFMO consumption for 20 weeks resulted in a marked reduction in PCNA protein expression in the dorsolateral prostate of TRAMP mice compared with the non-DFMO-fed TRAMP mice (Fig. 5A). The densitometric analysis of the bands exhibited a 32% decrease ($P < 0.001$) in PCNA protein expression in the DFMO-fed TRAMP mice when compared with the non-DFMO-fed TRAMP group (Fig. 5B). These results were further confirmed by immunohistochemical analysis (Fig. 6). Feeding of DFMO to nontransgenic littersmates did not result in any significant alterations in PCNA protein expression (Figs. 5 and 6).

**Effect of DFMO Consumption on Metastases Marker(s) in TRAMP Mice.** Uncontrolled progression of CaP leads to distant site metastases, which is responsible for the majority of CaP-related deaths in humans (40, 41). Therefore, we evaluated the effect of DFMO on the markers of CaP metastasis. The progression of CaP occurs via loss of functional expression of E-cadherin (42, 43), and the cadherin-catenin complex suppression and their loss from the epithelial cell have been described as increased invasiveness and...
H&E and examined at 40 magnification. A typical dorsolateral prostate from a non-
where a significant restoration of 246% (Fig. 5A). These results were further confirmed by immunohistochemical analysis where DFMO feeding to TRAMP mice resulted in the restoration of α- and β-catenin in the dorsolateral prostate of TRAMP mice (Fig. 6). In the nontransgenic littermates, however, DFMO supplementation was not found to result in noticeable alterations in the protein expression of these markers (Figs. 5 and 6).

**Fig. 4.** Effect of DFMO consumption on ODC enzyme activity and protein expression in TRAMP mice and their nontransgenic littermates. **A**, ODC enzyme activity in the dorsolateral prostate of nontransgenic (control), nontransgenic (DFMO-fed), TRAMP (control), and TRAMP (DFMO-fed) male mice from 8–28 weeks. A marked decrease in ODC enzyme activity was observed after DFMO feeding. Data from eight mice per group are shown here. **B**, ODC protein expression in the dorsolateral prostate of different nontransgenic and TRAMP groups. Representative data from four mice per group are shown here. **C**, densitometric analysis of ODC protein expression from the above blot indicates a marked decrease in protein expression after DFMO feeding. *, P < 0.001, Student’s t test, TRAMP (control) versus TRAMP (DFMO-fed). Bars, SE of eight mice.

**Effect of DFMO Cessation on Prostate Tumor Regrowth in TRAMP Mice.** The experiment was conducted to investigate whether the cessation of DFMO feeding causes a regrowth of CaP in TRAMP mice. As shown in Table 2, 20 weeks of DFMO feeding was effective in reducing CaP growth and invasion in TRAMP mice. The experimental animals, which received regular drinking water for 6

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**Fig. 3.** Histological examination of dorsolateral prostate in male TRAMP mice and their nontransgenic littermate after DFMO feeding. A, tissue sections were stained with H&E and examined at 40× magnification. A typical dorsolateral prostate from a nontransgenic mouse exhibited acini with abundant eosinophilic intralumenal secretions. DFMO feeding to a nontransgenic mouse did not exhibit any noticeable alterations. In TRAMP mice (control), extensive epithelial stratification with profound cribriform structures accompanied with marked thickening, remodeling, and hypercellularity of the fibromuscular stroma was observed. DFMO feeding to TRAMP mice resulted in a marked reduction in epithelial stratification and cribriform structures. B, a detailed examination of a DFMO-fed section (80×) reveals acini with many elongated hyperchromatic epithelial nuclei, with clumping of the chromatin and an increased nuclear:cytoplasmic ratio compared with the transgenic control at a similar magnification. Apoptotic cells were ascertained by morphological criteria of the cells examined under light microscopy. The details are described in the “Materials and Methods” section. Arrows, the apoptotic cells. C, a high apoptotic index was observed in the DFMO-fed group, which was exhibited by a significant increase in the number of apoptotic bodies and dense staining of the nuclei, compared with non-DFMO-fed TRAMP mice. *, P < 0.001, Student’s t test, TRAMP (control) versus TRAMP (DFMO-fed). Bars, SE.
weeks after 14 weeks of DFMO feeding, exhibit moderate local invasiveness in seminal vesicles and in the local prostate region. However, no distant site metastasis to lymph nodes and lungs was observed. The weight and volume of prostate and the GU apparatus was found to significantly increase ($P < 0.05$) in the experimental group, compared with DFMO-fed TRAMP mice. The histopathological examination of the dorsolateral prostate of the experimental group exhibit moderately differentiated prostate tumor where acini are completely filled with tumor yet still forming some glandular structures (data not shown).

DISCUSSION

The present investigation demonstrates the use of TRAMP mouse for CaP chemoprevention studies and shows that intervention with DFMO holds promise for use against CaP. For establishing proof of the principle of biomarkers assessing human CaP risk and for the translation of knowledge to humans, the chemopreventive potential of an agent should be validated in the animal models, which emulate progressive forms of human disease and where the cancer is developed without the need to administer unrealistic large doses of chemical carcinogens or hormones. In recent years, genetically manipulated animal models are emerging as resources for developing strategies against many pathological conditions, including cancer (13–15, 17, 18). For CaP, one such model is TRAMP where specific genes are involved in complex multifactorial process of prostate carcinogenesis. Furthermore, TRAMP mice that have an intact immune system and could serve as a biomarker for early detection of CaP in humans. Because ODC overexpression and accumulation of polyamines, $i.e.$, putrescine, spermine, and spermidine, are common features in most primary epithelial human cancers, such as cancers of skin (45), breast (46), and prostate (47), ODC could be exploited as a rational target for chemoprevention. In the present study, DFMO supplementation caused a rapid and substantial decrease in ODC enzyme activity and protein expression in the dorsolateral prostate with a significant decrease in hyperproliferation of the GU apparatus. Further, histopathological analysis was also found to correlate with the biochemical response as well as the proliferation. The other significant finding of this study is the apoptotic index, which was found to be significantly greater in DFMO-fed TRAMP mice. This result suggests that in addition to inhibiting proliferation, apoptosis plays a significant role in the DFMO inhibition of the growth of CaP in TRAMP mice. These findings are in accordance with recent studies (48, 49) where DFMO-induced apoptosis is shown. These results support our hypothesis that ODC could be used as a rational target and DFMO as an ideal agent for chemoprevention in prostate carcinogenesis.

Invasion of CaP to distant sites causes metastatic disease that is regarded as the major cause of CaP-related deaths in humans (40, 41). Tumor cells acquire this increased invasive potential by a complex pathway, which include decreased cell substrate attachment and cell-cell adhesion, as well as increased cell motility. In the TRAMP model, loss of E-cadherin has been suggested to play a major role in modulating metastasis (42, 43). Many studies have shown that the cadherin-catenin complex is correlated with the loss of cellular differentiation and acquisition of invasive and metastatic potential in human tumors, including head and neck (50, 51), breast (52, 53) bladder (54), gastric (55), prostate (56), colon (57), and basal cell carcinoma of the skin (58). We have demonstrated that DFMO feeding to TRAMP mice
Immunohistochemical analysis of proliferation and metastases markers in TRAMP mice and their nontransgenic littermates after DFMO feeding. Immunohistochemical detection of mPB-1, PCNA, E-cadherin, and \(\alpha\) and \(\beta\)-catenin in the dorsolateral prostate of nontransgenic (control), nontransgenic (DFMO-fed), TRAMP (control), and TRAMP (DFMO-fed) male mice from 8–28 weeks. A representative figure from each group is shown here. In TRAMP mice, an extensive mPB-1 staining was observed in the lumen and acinar regions of the epithelium, whereas extensive PCNA staining was observed in the nuclei. DFMO feeding resulted in the marked reduction in mPB-1 and PCNA staining in these mice. The control as well as DFMO-fed nontransgenic animals did not exhibit mPB-1 expression, whereas few PCNA stained cells were observed in these groups. Normal E-cadherin and \(\alpha\) and \(\beta\)-catenin expression was maintained in the basolateral cell-cell borders of the epithelium in nontransgenic control and DFMO-fed animals. In the TRAMP mouse, a significant loss in the normal expression of E-cadherin and \(\alpha\) and \(\beta\)-catenin was observed in the dorsolateral prostate, whereas DFMO feeding resulted in a significant restoration of these proteins.

Table 2  Effect of DFMO cessation on the morphology of prostate and GU weight in TRAMP mice

<table>
<thead>
<tr>
<th>Group(^b)</th>
<th>No. of Animals</th>
<th>CaP</th>
<th>Lymph</th>
<th>Lungs</th>
<th>Prostate weight (mg)</th>
<th>Prostate volume (mm(^3))</th>
<th>GU weight (g)</th>
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<tbody>
<tr>
<td>Control (No DFMO)</td>
<td>6</td>
<td>Severe</td>
<td>6/6 (100%)</td>
<td>4/6 (66.6%)</td>
<td>68.6 ± 8.86</td>
<td>-</td>
<td>-</td>
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<tr>
<td>DFMO feeding for 20 wk</td>
<td>6</td>
<td>Mild</td>
<td>0/6 (0%)</td>
<td>0/6 (0%)</td>
<td>28.2 ± 2.84</td>
<td>17.54 ± 1.66</td>
<td>1.02 ± 0.12</td>
</tr>
<tr>
<td>DFMO feeding (14 wk), followed by water (6 wk)</td>
<td>6</td>
<td>Moderate</td>
<td>0/6 (0%)</td>
<td>0/6 (0%)</td>
<td>38.8 ± 4.48(^c)</td>
<td>24.22 ± 2.12(^d)</td>
<td>1.83 ± 0.26(^d)</td>
</tr>
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\(^a\) The data represent the mean ± SE of six mice.
\(^b\) Mice (8 weeks of age) were given plain drinking water (for control group) or DFMO in drinking water for 20 or 14 weeks. For the 14-week study, the animals were returned to plain drinking water after 14 weeks. At the age of 28 weeks, the animals were sacrificed and studied for prostate tumorigenesis and metastasis.
\(^c\) Metastasis in the lymph was examined microscopically, whereas metastasis in lungs was examined by using the India ink method as described in “Materials and Methods.”
\(^d\) \(P < 0.05, \) DFMO-fed (for 20 weeks) TRAMP versus DFMO-fed (for 14 weeks), Student’s \(t\) test.
caused a significant restoration of the loss of E-cadherin and α- and β-catenin. We suggested that this might be a reason for the complete absence of metastases in these mice.

The biology of CaP includes a well-differentiated histopathological and more benign malignant behavior. TRAMP mice exhibit PB-Tag transgene expression in prostate cell epithelium. This is where cancers arise from normal cells, in their microtissue environment, and progress through multiple stages, as does human cancer (22, 24). Our studies with DFMO chemoprevention have shown a marked decrease in development and progression of CaP in TRAMP mice. It is important to emphasize that DFMO treatment to TRAMP mice did not alter the expression of the PB-Tag transgene because it was readily detectable in both DFMO and non-DFMO-fed groups (data not shown). Further, the expression of the PB-Tag transgene has been shown to precede the histological appearances of carcinoma in the TRAMP mice, as in other transgenic models, suggesting that the proliferative response as a consequence of Tag oncoprotein expression is a prerequisite but not sufficient for neoplastic transformation (24). These observations support the hypothesis that cells expressing the transgene are in a preneoplastic state and that other stochastic events are required to confer proliferation and ultimately a malignant state.

The use of DFMO as a cancer chemopreventive agent has been strengthened in recent years. Several reports have shown that oral administration of DFMO to tumor-bearing animals for ≥2 weeks reduces the frequency and development of pulmonary and liver metastasis and concomitantly inhibits the growth of primary tumors (59–62). DFMO was subsequently shown to inhibit carcinogen-induced cancer development in a number of rodent models (59–62). Parallel to these studies, our results demonstrate that DFMO was able to suppress tumor development and progression in the TRAMP mouse as long as DFMO was continuously administered. However, when DFMO feeding was suspended (at 14 weeks), an increased local invasiveness and CaP growth were observed after 6 weeks in TRAMP mice, further suggesting that ODC could be used as a target as well as a biomarker for CaP chemoprevention. DFMO treatment has been reported to exhibit ototoxicity in humans (63). However, recent clinical cancer chemoprevention trials indicate that low doses of DFMO can be given for a longer period to cancer patients without any detectable ototoxicity or other side effects (38, 60, 64). It is important to emphasize that the feeding regimen of DFMO used in this study did not exhibit any toxic symptoms in TRAMP mice or in their nontransgenic littermates.

In summary, the chemoprevention studies by DFMO in TRAMP mice were effective, and all of the markers of proliferation examined nicely correlated with tumor invasiveness that was affected by DFMO. We conclude that ODC represents a promising and rational target for CaP chemoprevention, and TRAMP mice are excellent models for screening of novel drugs and CaP chemopreventive regimens for potential human use.

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Chemoprevention of Prostate Carcinogenesis by $\alpha$-Difluoromethylornithine in TRAMP Mice

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