INTRODUCTION

Advances in molecular biology and genetics combined with ongoing clinical research have identified epigenetic factors or inherited mutations predisposing patients to malignancy. As such, there is a growing role for prevention regimens to treat high-risk individuals with either systemic predisposition for cancer development or specific high-grade neoplastic precursor lesions (1, 2). A time-consuming aspect of chemoprevention trials is the choice of endpoints. If reduction in visible malignancy is the goal, these studies may require years with either systemic predisposition for cancer development or specific high-grade neoplastic precursor lesions (1, 2). A time-consuming aspect of chemoprevention trials is the choice of endpoints. If reduction in visible malignancy is the goal, these studies may require years

ABSTRACT

To be informative for chemoprevention, animal models must both closely emulate human disease and possess surrogate endpoint biomarkers that facilitate rapid drug screening. This study elucidated site-specific histopathological and biochemical surrogate endpoint biomarkers of spontaneous epidermal carcinogenesis in K14-HPV16 transgenic mice and demonstrated that the incidence and severity of these markers were decreased by the ornithine decarboxylase (ODC) inhibitor difluoromethylornithine (DFMO). The cumulative incidence of visible epidermal cancers in 127 untreated transgenic mice was 42% by 52 weeks of age, most frequently affecting the chest as flat lesions in association with chronic ulcers, or in the ear as protuberant masses. Microscopic malignancies were detected in 39% of 32-week-old transgenic mice and were found to emerge from precursor lesions that were of two distinct types: dysplastic sessile ear papillomas and hyperproliferative follicular/interfollicular chest dysplasias. ODC activity and tissue polyamine contents were differentially elevated in ear and chest skin during carcinogenesis, such that there was a marked elevation of both parameters of polyamine metabolism as early as 4 weeks of age in the ear, whereas in the chest, polyamine metabolism was increased significantly only in the late stages of neoplastic progression and in epidermal cancers. Administration of 1.0% DFMO in the drinking water from 4 to 32 weeks of age prevented both visible and microscopic malignancies and significantly decreased the incidence of chest and ear precursor lesions. ODC activity and tissue putrescine content were markedly diminished by DFMO chemoprevention in ear skin, whereas there was a more modest decline of these parameters in chest skin. DFMO treatment of transgenic mice from 28 to 32 weeks of age was associated with an absence of ear cancer and a marked regression of dysplastic papillomas. In contrast, the results in chest skin were complex in that the severity of chest precursors diminished, but their incidence was unchanged, and microscopic cancers were still detectable within these lesions. Collectively, this study highlights the utility of multistage epidermal carcinogenesis in K14-HPV16 transgenic mice both for the study of the biology of, and as a screening tool for, novel drugs and chemopreventive regimens.

DFMO treatment of transgenic mice from 28 to 32 weeks of age was associated with an absence of ear cancer and a marked regression of dysplastic papillomas. In contrast, the results in chest skin were complex in that the severity of chest precursors diminished, but their incidence was unchanged, and microscopic cancers were still detectable within these lesions. Collectively, this study highlights the utility of multistage epidermal carcinogenesis in K14-HPV16 transgenic mice both for the study of the biology of, and as a screening tool for, novel drugs and chemopreventive regimens.

INTRODUCTION

Advances in molecular biology and genetics combined with ongoing clinical research have identified epigenetic factors or inherited mutations predisposing patients to malignancy. As such, there is a growing role for prevention regimens to treat high-risk individuals with either systemic predisposition for cancer development or specific high-grade neoplastic precursor lesions (1, 2). A time-consuming aspect of chemoprevention trials is the choice of endpoints. If reduction in visible malignancy is the goal, these studies may require years for completion in humans and months in rodent models. Identification of SEBs (3), clinical, histological, or molecular milestones detectable at characteristic stages of neoplastic progression, allows elucidation of chemopreventive efficacy in shorter time intervals, enhancing throughput in the development of novel drugs (4, 5). Thus, there is an imminent need for animal models that both recapitulate the multistage nature of human cancer and possess informative SEBs for drug screening (6).

A variety of animal models have been used for prevention research (6). A frequently used paradigm is drug administration concomitant with, or subsequent to, exposure of rodents to chemical carcinogens (7–17). In general, these experiments investigate the biology of chemoprevention at the last stages of premalignant neoplasia, or end-stage cancers. Growth of human cancer xenografts in immunodeficient mice is another screening system (18, 19). However, it is increasingly apparent that neoplastic lesions at earlier stages of carcinogenesis respond differently to chemopreventive agents compared with established malignancies used in xenograft assays (3–5).

Transgenic and knockout mouse models are emerging resources for chemoprevention research. Advantages of genetically manipulated mouse include induction of carcinogenesis by discrete genetic chang(e)s (20) and the ability to overexpress oncogenes or ablate tumor suppressor genes implicated in human cancers (21). Both of these features provide an opportunity to link efficacy of chemopreventive agents to specific biochemical or genetic pathways. One notable example is the marked reduction in the number of intestinal polyps induced by nonsteroidal anti-inflammatory drugs, which inhibit the enzymatic activity of COX-2, in APCMin and APCΔ761 knockout mice (22, 23). Two distinct genetic lesions can also be produced in one animal by intercrossing either two transgenic or transgenic and knockout mice, creating closer replicas of clinical disease (24, 25) and presenting the opportunity to further characterize molecular mechanisms underlying chemoprevention. Thus, the importance of COX-2 inhibition in chemoprevention of intestinal carcinogenesis was bolstered by the significant reduction in polyt incidence in APCΔ761 and COX-2 double knockout mice, recapitulating the phenotype induced by pharmacological COX-2 inhibition (26).

To investigate the utility of a transgenic model of spontaneous epidermal carcinogenesis for chemoprevention studies, we used K14-HPV16 transgenic mice, which express the oncogenes of the “high-risk” HPV type 16 in basal squamous epithelium and develop spontaneous multistage epidermal carcinogenesis that shares both histological and molecular features with HPV-associated human cancers (27–29). The “suicide” ODC inhibitor, DFMO, was chosen for chemoprevention because of its documented activity both in murine epidermal chemical carcinogenesis models (17, 30, 31) and in ODC transgenic mice (32, 33). Detailed investigation of epidermal neoplastic progression in untreated K14-HPV16 transgenic mice uncovered

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

3 The abbreviations used are: SEB, surrogate endpoint biomarker; DFMO, difluoromethylornithine; BrdUrd, bromodeoxyuridine; K14, keratin-14, HPV16, human papillomavirus type 16; ODC, ornithine decarboxylase; ORS, outer root sheath; COX-2, cyclooxygenase type 2; APC, adenomatous polyposis coli; TUNEL, terminal transferase uridine end labeling.
site-specific histopathological lesions, sessile dysplastic ear papillomas, and hyperproliferative follicular/interfollicular chest dysplasias that appeared to be immediate precursors to invasive malignancies. Moreover, biochemical analysis of neoplastic transgenic skin revealed distinctive increases in ODC activity and tissue polyamine content during spontaneous carcinogenesis in chest and ear skin and a different response to DFMO at each site. In addition, the phenotypic and histopathological response to DFMO chemoprevention was distinctive in ear versus chest skin, such that DFMO appeared to inhibit papilloma formation in the ear, whereas in the chest, transgene-induced expansion and hyperproliferation of the hair follicle ORS was diminished. Thus, this study suggests that different sites of squamous carcinogenesis may have distinctive biology of neoplastic progression and particular sensitivities to chemoprevention and highlights the use of K14-HPV16 transgenic mice as tools for screening novel chemopreventive agents.

**MATERIALS AND METHODS**

**Transgenic Mice.** Transgenic mice used in this study were from the K14-1203#1 line, containing a wild-type version of the HPV early region cloned behind the human keratin-14 enhancer/promoter (27–29, 34, 35). K14-1203#1 mice had been serially backcrossed into the FVB/n inbred strain for n = 21–22 generations prior to use in these experiments. Throughout the remainder of this report, K14-1203#1 mice are referred to as K14-HPV16 transgenic mice.

**Study Design, DFMO Treatment, and Verification of DFMO Activity.** Fig. 1 details the study design. The incidence of carcinoma was prospectively analyzed in a cohort of 127 K14-HPV16 mice observed from birth to 52 weeks of age. The biology of epidermal carcinogenesis was investigated by analyzing untreated transgenic mice at 4, 8, 16, and 32 weeks of age (Fig. 1). These untreated transgenic mice were used as concurrent controls in the chemoprevention trial. The effect of chemoprevention was studied by serial sacrifice of transgenic mice treated with DFMO from 4 to 8, 4 to 16, and 4 to 32 weeks of age. Additional untreated and treated nontransgenic controls were included at each of these time points (Fig. 1). The effect of DFMO on established, high-grade, epidermal precursor lesions and microinvasive squamous carcinomas was investigated by treating transgenic mice from 28 to 32 weeks of age. DFMO was administered as a 1.0% solution in acidified drinking water, and its concentration was verified by high-pressure liquid chromatography analysis.

**Tissue Processing and Histopathology.** Mice were weighed and anesthetized with 37.5 mg/kg of a 0.25% Avertin solution (38) and perfused via the aortic root with a 3.75% (pH 7.0) solution of freshly prepared paraformaldehyde in PBS. Visible cancers were removed with a 2–3 mm margin of adjacent skin and bisected. Two different 10 × 2-mm pieces of chest skin were obtained in either transverse (coronal), or vertical (cephalo-caudal) orientations. The transverse chest sample bisected ulcerated or malignant lesions but was perpendicular to the hair follicles, whereas the vertical sample paralleled the hair follicles. The ear sample included at least 3 mm of distal ear. Skin samples were postfixed in 3.75% paraformaldehyde overnight at 4°C. Tissues were paraffin embedded, sectioned, and stained with hematoxylin and eosin as described previously (27). Two adjacent 5-μm sections were routinely obtained from ear and chest skin for histopathology. To minimize sampling error and to maximize detection of occult cancers, tissue samples were always obtained from chest and ear regions most visibly affected by ulceration, thickening, redness, or papillomatosis. Moreover, up to 10 adjacent microscopic sections were taken through chest or ear precursor lesions, from both untreated and DFMO-treated transgenic mice, to search for microinvasive carcinomas. To control for observer bias, independent analysis was performed on coded slides by a veterinary pathologist blinded to the treatment status of the transgenic mice.

**BrdUrd Incorporation.** Mice were injected with 100 mg/kg of BrdUrd i.p. and sacrificed 2 h later as described above; the tissues were postfixed in 3.75% paraformaldehyde for 12–18 h. Immunoperoxidase staining for detection of BrdUrd-labeled nuclei was performed on 5-μm paraffin sections as described previously, except that the protease digestion time was shortened to 25–30 s (27). The BrdUrd labeling index was determined by counting 1000 nuclei in sequential ×20 fields and dividing the number of labeled nuclei by the total number of nuclei. In mice with large histopathological variation in the extent of epidermal thickening and cellularity, the three most affected and three least affected fields were counted, and a combined mean score was used in the analysis. Three to four nontransgenic and four to eight transgenic mice were analyzed at each time point during spontaneous carcinogenesis or during chemoprevention (Fig. 1). There were no statistical differences between nontransgenic mice either with or without DFMO treatment or at any time interval in the study; therefore, the results of BrdUrd incorporation were pooled for this entire group (n = 13).

**TUNEL Staining.** A modification of the TUNEL assay (39) with fluorescein-concentration detection (Oncor S7110, Gaithersburg, MD) was used in this study. Briefly, 5-μm sections were baked at 35°C for 10 min, immediately deparaffinized in xylene, rehydrated, and equilibrated in PBS. Sections were digested with 5 μg/ml proteinase K dissolved in PBS for 20 min at 37°C. The reaction
was stopped by using running tap water, and following equilibration in PBS, the sections were postfixed in 3.75% paraformaldehyde for 15 min. After extensive washes in water, the sections were incubated with an equilibration buffer (Oncor) for 5 min and then incubated with a 1:10 dilution of terminal transferase in reaction buffer (Oncor). Dilution of the terminal transferase enzyme was critical to avoid background labeling. The remainder of the protocol proceeded according to the manufacturer’s instructions (Oncor). TUNEL-positive cells were visualized using ×50 or ×63 oil immersion using FITC emission (see below).

**Keratin Immunohistochemistry.** Keratin immunohistochemistry was performed on paraffin-embedded sections using methods described previously (27, 28). Sections were incubated with the following antibodies: mouse keratin-14, -5, and -6 at 1:2000 (BAbCo prb-155p, 160p, and 169p, respectively; Berkeley, CA), mouse keratin-10 at 1:1000 (BAbCo prb-159p), loricrin at 1:10,000 (BAbCo prb-145p), and processed for two-stage immunoperoxidase detection as described previously (27).

**mRNA in Situ Hybridization.** In situ hybridization for visualization of HPV16 E6/E7 mRNA was accomplished using a 35S-labeled riboprobe as described previously (35, 36). Sections were exposed for 1 month at 4°C prior to development.

**Determination of Skin ODC Activity and Polyamine Content.** Biopsies of chest and ear skin were obtained from 3 to 6 untreated transgenic mice at 4, 8, 16, and 32 weeks of age. 13 untreated or treated nontransgenic mice, and 3 transgenic mice treated with DFMO from 4 to 32 weeks of age. The data from the nontransgenic mice were pooled because there was no effect of treatment on ODC or tissue polyamine content. Skin samples were snap frozen in liquid nitrogen and stored at −80°C until analysis. Biopsies were also obtained from four different chest cancers and processed similarly. ODC activity was determined on whole skin samples as described recently (40). Tissue content of putrescine, spermidine, and spermine were determined by high-pressure liquid chromatography (40, 41). Similar to the BrdUrd analysis, the ODC activities and polyamine contents of the entire group of nontransgenic mice were pooled.

**Image Analysis.** Sections were visualized on a Leica DM HT microscope. Images were captured with an attached air-cooled CCD camera (SPOT camera; Diagnostic Instruments, Sterling Heights, MI) linked to a PC computer. Images and figures were composed using Adobe Photoshop 5.0 (Adobe Systems, San Jose, CA).

**Statistical Analysis.** Numerical data are reported as mean ± SD. Statistical analysis included the Mann-Whitney U test, 2-way ANOVA, and $\chi^2$ analysis. Statistical calculations and significance determinations were performed using a commercial software package (Graph Pad Prism 2, San Diego, CA).

**RESULTS**

**Phenotypic and Histopathological Analysis of Epidermal Carcinogenesis in K14-HPV16 Transgenic Mice.** Previous work, including our own studies, demonstrated the susceptibility of the FVB/n in-bred mouse strain to epidermal carcinogenesis. Since these prior studies were performed at a backcross of n = 12–14 into FVB/n, we investigated the incidence of overt epidermal squamous cancer in 127 K14-HPV16 transgenic mice observed from birth to 52 weeks of age at a backcross of n = 21–22 into FVB/n (Fig. 1). Visible epidermal cancers developed in 54 (42%) transgenic mice by 52 weeks of age. Malignancies appeared from 16 to 52 weeks of age (mean, 36 ± 14 weeks), most frequently in chest skin (Table 1), and often emerging from the edges of chronic ulcers (data not shown). The second most frequent sites of cancer occurrence were ear or truncal skin exclusive of chest, with occasional skin cancers appearing on the face (Table 1). Because marked biological and histopathological differences were particularly evident between chest and ear malignancies, we focused our subsequent studies on carcinogenesis at these two sites. Chest cancers were predominantly flat lesions with heaped-up margins, occasionally metastasizing to regional lymph nodes. Ear cancers were protuberant lesions, capped by keratin horns, that were locally invasive but did not metastasize. Histopathologically, chest cancers were moderate to poorly differentiated, whereas ear cancers were well-differentiated squamous carcinomas (28).

To investigate whether the model possessed microscopic SEBs characteristic of specific stages of neoplastic progression, we undertook a detailed histopathological investigation of chest and ear skin from untreated transgenic mice at intervals from 4 to 32 weeks of age (Fig. 1). At 32 weeks of age, microscopic chest lesions were detected that contained a corrugated surface and extensive expansion of both the interfollicular epidermis and the entire hair follicle ORS, encompassing the bulge and surrounding the sebaceous glands, which were also hyperplastic (Fig. 2C). Similar to visible malignancies, these chest lesions were frequently adjacent to ulcers (data not shown). High-power views revealed dysplastic keratinocytes with enlarged hyperchromatic nuclei dispersed throughout the ORS and interfollicular epidermis, microulcerations (Fig. 2E, arrowhead), and extensive parakeratotic replacement of the normally anucleate stratum corneum with nucleated keratinocytes (Fig. 2E). This chest histopathology was designated as hyperproliferative follicular/interfollicular dysplasia to include each critical aspect of these lesions. Microscopic squamous carcinomas were also detected emerging directly from the junction of hyperproliferative follicular/interfollicular dysplasias and larger areas of chest ulceration (Fig. 2G). In ear skin of 32-week-old transgenic mice, sessile papillomas were detected containing foci of markedly enlarged basal and suprabasal keratinocytes with massive, hyperchromatic nuclei, consistent with carcinoma in situ or high-grade dysplasia (Fig. 2D). Sessile dysplastic papillomas also contained foci of microinvasive cancer, with keratinocytes breaking through the basement membrane and invading the underlying dermis (Fig. 2F). Taken together, 39% of transgenic mice developed microscopic or microinvasive chest and ear cancers at 32 weeks of age. 21% developed microscopic chest malignancies, and 18% developed microscopic ear malignancies (Table 2). Detection of microscopic cancers within hyperproliferative follicular/interfollicular chest dysplasias and sessile dysplastic ear papillomas supported the hypothesis that these complex pathologies were more immediate antecedents to malignancy than flat dysplasia or hyperplasia. Therefore, these lesions were further designated as chest or ear “precursors.” In 32-week-old transgenic mice, chest precursors were present in 64%; and ear precursors were present in 82% (Table 2).

<table>
<thead>
<tr>
<th>Anatomic site</th>
<th>Total</th>
<th>Chest</th>
<th>Ear</th>
<th>Trunk</th>
<th>Head</th>
</tr>
</thead>
<tbody>
<tr>
<td>54*</td>
<td>33</td>
<td>9</td>
<td>9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>63**</td>
<td></td>
<td>(61)</td>
<td>(17)</td>
<td>(17)</td>
<td>(6)</td>
</tr>
</tbody>
</table>

* Total number of mice bred was 127.
** Percentage of mice bred.
* Percentage of cancers at each site.

<table>
<thead>
<tr>
<th>Percentage of cancers at each site.</th>
<th>Anatomic site</th>
<th>Total</th>
<th>Chest</th>
<th>Ear</th>
<th>Trunk</th>
<th>Head</th>
</tr>
</thead>
<tbody>
<tr>
<td>54*</td>
<td>33</td>
<td>9</td>
<td>9</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>63**</td>
<td>(61)**</td>
<td>(17)</td>
<td>(17)</td>
<td>(6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Total number of mice bred was 127.
** Percentage of mice bred.
* Percentage of cancers at each site.
Phenotypic and Histopathological Effects of DFMO Chemoprevention Initiated Prior to Dysplasia.

To investigate the biology of chemoprevention administered at the initial stages of carcinogenesis, we studied the effects of DFMO on chest and ear skin histopathological SEBs from transgenic mice treated for 4–8, 4–16, or 4–32 weeks of age (Fig. 1). DFMO chemoprevention from 4 to 8 weeks of age did not affect the incidence, extent, or severity of hyperplasia and dysplasia of the interfollicular epidermis in either chest or ear skin (data not shown). However, DFMO chemoprevention from 4 to 16 weeks of age induced discernable effects in both ear and chest skin. In the chest, expansion of the hair follicle ORS was less pronounced in DFMO-treated transgenic mice compared with untreated transgenic controls (data not shown). However, the incidence of chest precursor lesions was not different in treated compared with untreated transgenic mice (Table 2). In the ear, papillomatosis and dermal inflammatory cell infiltration were essentially prevented by DFMO treatment from 4 to 16 weeks of age (Table 2). The most striking features of chemoprevention were evident in transgenic mice treated from 4 to 32 weeks of age (Table 2 and Fig. 3). There was a complete absence of microscopic malignancies in either chest or ears in transgenic mice (Table 2). Moreover, sessile dysplastic ear papillomas were almost entirely prevented by DFMO, and the incidence of dysplastic follicular/inter-
fOLLICULAR CHEMOPREVENTION OF EPIDERMAL CANCER IN TRANSGENIC MICE

Table 2  Effect of chemoprevention on skin pathology in K14-HPV16 transgenic (TG) mice

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Chest</th>
<th>Ear</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ulcer</td>
<td>Hyperproliferative follicular/interfollicular dysplasia</td>
</tr>
<tr>
<td>TG Control 16 wk</td>
<td>6 (28)*</td>
<td>4 (19)</td>
</tr>
<tr>
<td>(n = 21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG DFMO 16 wk</td>
<td>10 (38)</td>
<td>4 (15)</td>
</tr>
<tr>
<td>(n = 26)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG Control 32 wk</td>
<td>12 (43)</td>
<td>18 (64)</td>
</tr>
<tr>
<td>(n = 28)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG DFMO 4–32 wk</td>
<td>14 (54)</td>
<td>5b (19)</td>
</tr>
<tr>
<td>(n = 26)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG DFMO 28–32 wk</td>
<td>15 (56)</td>
<td>13 (48)</td>
</tr>
<tr>
<td>(n = 27)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG Control 16 wk</td>
<td>18 (86)</td>
<td>18 (86)</td>
</tr>
<tr>
<td>(n = 26)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG DFMO 16 wk</td>
<td>2b (8)</td>
<td>0b</td>
</tr>
<tr>
<td>(n = 26)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG Control 32 wk</td>
<td>18 (64)</td>
<td>23 (82)</td>
</tr>
<tr>
<td>(n = 28)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG DFMO 4–32 wk</td>
<td>5b (19)</td>
<td>1b (4)</td>
</tr>
<tr>
<td>(n = 26)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG DFMO 28–32 wk</td>
<td>11 (41)</td>
<td>4b (15)</td>
</tr>
<tr>
<td>(n = 27)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Number and percentage of mice with respective lesion.

b P < 0.05, untreated, control transgenic compared with DFMO-treated transgenic mice, Mann-Whitney U test.

follicular chest lesions was significantly decreased (Table 2). Histologically, both chest (Fig. 3, A, C, and E) and ear skin (Fig. 3, G and I) displayed characteristic microscopic phenotypes associated with DFMO chemoprevention. In the chest, enlargement of the hair follicle ORS was restricted to the infundibular region immediately below the interfollicular epidermis (Fig. 3C), whereas the cellularity of the ORS encompassing the bulge region and surrounding the sebaceous glands was reduced to normal (Fig. 3A and high-power view in Fig. 3C). DFMO also induced cystic degeneration of the sebaceous gland and the hair follicle inner root sheath (Fig. 3, A and C) and nearly eliminated the presence of nucleated cells in the stratum corneum (Fig. 3C). Despite considerable activity in the hair follicle, DFMO did not affect hyperplasia of the interfollicular epidermis (Fig. 3, A and C). Persistence of abnormal epidermis was responsible for an incidence of chest microulceration that was similar to untreated transgenic mice. However, DFMO appeared to alter the histopathology of epithelial elements within ulcers, such that hair follicle remnants within ulcers were evident as differentiated cystic structures (Fig. 3E). In the hair, DFMO chemoprevention from 4 to 32 weeks of age essentially prevented papillomatosis (Fig. 3G) and reduced both corneal parakeratosis and dermal inflammatory cell infiltration (Fig. 3, G and I). Despite activity against ear precursors, both hyperplasia and dysplasia were still present in ear epidermis after 4–32 weeks of DFMO chemoprevention (Fig. 3I).

DFMO treatment from 4 to 32 weeks also affected body weight. DFMO-treated, nontransgenic mice were 15 g lighter than untreated controls, 30 ± 4 versus 45 ± 9 g. In contrast, transgenic mice treated with DFMO were only 2 g lighter, 23 ± 3 versus 25 ± 3 g, than untreated transgenic controls. The difference between treated and untreated transgenic mice, although slight, was statistically significant at P < 0.05. Subsequent to these data, follow-up studies of both nontransgenic and transgenic mice treated with DFMO for shorter intervals, 4–8 or 4–16 weeks, did not reveal differences in body weights between treated and untreated mice (data not shown).

DFMO Treatment of Transgenic Mice with Established Epidermal Neoplasia. Clinically, advanced neoplastic lesions may be detected that are known to be at risk for development of invasive cancer, or patients may have a history of long-term exposure to carcinogens with an epithelium subsequently at risk for malignant conversion due to accumulated somatic mutations (42). To model these clinical scenarios, K14-HPV16 transgenic mice were treated with DFMO from 28 to 32 weeks of age, designated as “late-stage treatment,” then sacrificed for histopathological analysis of ear and chest skin (Fig. 3, B, D, F, and H; Table 2). Late-stage treatment completely prevented visible and microscopic ear cancers (Table 2). Ear papillomas visibly regressed and were not detectable microscopically after late-stage treatment (Fig. 3F). Despite regression of precursor lesions, ear epidermal hyperplasia and dysplasia persisted (Fig. 3H), as did infiltration of the dermis with inflammatory cells, fibroblasts, and capillaries (Fig. 3, compare F and H with G and I). In chest skin, the incidence of dysplastic follicular/interfollicular lesions was not significantly reduced (Table 2) by late-stage treatment. Although the degree of the ORS expansion was decreased by late-stage treatment compared with untreated 32-week-old transgenic mice (compare Fig. 3B with Fig. 2C), the ORS was still enlarged compared with transgenic mice treated with DFMO from 4 to 32 weeks of age (Fig. 3, compare A with B). Moreover, the incidence of microscopic chest malignancies was only decreased to 50% of untreated transgenic controls but not eliminated by late-stage treatment (Fig. 3D).

In vivo BrdUrd Incorporation during Spontaneous Carcinogenesis and DFMO Chemoprevention. Since previous studies suggested DFMO was an antiproliferative agent (43, 44) and our previous work documented increased epidermal keratinocyte DNA synthesis in the ears of K14-HPV16 transgenic mice during carcinogenesis (45), in vivo BrdUrd incorporation was determined in untreated and DFMO-treated K14-HPV16 transgenic mice at each stage of neoplastic progression (Figs. 1, 4, and 5). In untreated transgenic mice, baseline BrdUrd incorporation was significantly elevated in both ear and chest epidermis in 4-week-old transgenic compared with nontransgenic mice (Fig. 4). There was a trend toward incremental increase in BrdUrd incorporation at each stage of epidermal neoplasia, but these increases beyond the 4-week time were not statistically significant (Fig. 4). DFMO chemoprevention from 4 to 32 weeks of age significantly decreased BrdUrd incorporation in both chest and ear epidermis (Fig. 4). In the chest, 4–32 weeks of DFMO chemoprevention reduced frequency of BrdUrd-positive keratinocytes in the hair follicle ORS, which was in marked contrast to the hair follicle ORS of untreated 32-week-old transgenic mice, which contained numerous S-phase keratinocytes (Fig. 5). DFMO also decreased BrdUrd incorporation after 4–16 weeks of chemoprevention, but this reduction was only significant in the ear skin (Fig. 4). Despite reduction in BrdUrd incorporation induced by DFMO, keratinocyte S-phase fraction remained elevated in transgenic ear and chest epidermis compared with nontransgenic mice at each duration of treatment (Fig. 4). Moreover, DFMO had no effect on BrdUrd incorporation in transgenic mice treated from 4 to 8 weeks of age (Fig. 4).

Response of Tissue ODC Activity and Polyamine Content to Spontaneous Carcinogenesis and DFMO Chemoprevention. Increased ODC activity and overall expansion of polyamine pools have been documented in rodent models of epidermal carcinogenesis in-
duced by chemical carcinogens (16), (31). As such, two goals of our study were to investigate alterations of ODC activity and tissue polyamine content during spontaneous epidermal carcinogenesis in the K14-HPV16 transgenic mice and to study the effects of DFMO chemoprevention on polyamine metabolism during neoplastic progression.

In the ear, an increase in ODC activity was evident as early as 4 weeks of age (Fig. 6), corresponding to the visible hyperplastic phenotype at that time point. Further increases in ODC activity occurred as neoplasia progressed (Fig. 6). ODC activity in chest skin of transgenic mice also increased at 8 and 16 weeks of age. However, elevations of ODC activity did not reach statistical significance until 32 weeks of age (Fig. 6), the stage of epidermal carcinogenesis wherein hyperplastic interfollicular/follicular dysplastic precursors and microinvasive cancers had developed (Table 2 and Fig. 2). A further marked increase in ODC activity occurred in chest cancers (Fig. 6). Increases in the tissue contents of putrescine and spermidine paralleled elevations in ODC activity in both ear and chest (Fig. 6).

Concurrent, untreated nontransgenic controls were sacrificed at each of these stages of neoplastic progression. Because ODC activity in chest and ear skin of nontransgenic control skin was statistically similar at each time point, the results over time were pooled for the

Fig. 3. Effect of DFMO chemoprevention on chest and ear skin of transgenic mice. Histopathological response of chest (A, C, and E) and ear (G and I) skin to DFMO chemoprevention from 4 to 32 and 28 to 32 (B and D, chest; F and H, ear) weeks of age. In the chest, DFMO eliminated hyperplasia of the hair follicle ORS (A, arrowhead) and diminishing hypertrophy of the sebaceous glands by apparent cystic degeneration (A, arrow). A high-power view of a hair follicle from a DFMO-treated mouse demonstrates cystic destruction of the sebaceous glands, enlargement of the ORS limited to the upper third of the follicle, and persistent interfollicular dysplasia (C). In chest ulcers, DFMO induced cystic changes in isolated hair follicle elements (E). DFMO chemoprevention prevented papilloma formation in the ear and reduced dermal inflammatory cell infiltration (G and I) but did not diminish the severity or frequency of dysplastic foci in transgenic mice (I). DFMO treatment from 28 to 32 weeks, late-stage treatment, diminished but did not eliminate ORS hyperplasia (B, arrowhead) or sebaceous gland hypertrophy (B, arrow) in the chest. Microscopic cancer was also still detectable in late-stage treated transgenic mice (D). In the ear, the incidence of papillomas was markedly reduced by late-stage therapy (F), but dermal inflammatory infiltration (F and H) and dysplasia were persistent and unaffected by therapy (H). Bars: A, B, D, E, and G, 100 μm; C, H, and I, 10 μm.
entire group for comparison with those of transgenic mice at each stage of progression (data not shown). The effects of DFMO chemoprevention on ODC activity and tissue polyamine content during epidermal neoplastic progression were analyzed in skin from transgenic mice treated with DFMO from 4 to 32 weeks of age (Fig. 7). The greatest response was in the ear, where DFMO chemoprevention decreased ODC activity to levels similar to nontransgenic mice (Fig. 7). In contrast, there was only a 26% reduction in ODC activity in chest skin during DFMO chemoprevention (Fig. 7). Tissue putrescine content was also decreased to levels of nontransgenic mice in both ear and chest transgenic skin by DFMO treatment (Fig. 7). In contrast, spermidine levels were decreased by only 26 – 28%, whereas spermine levels increased 20 – 30% (Fig. 7) during chemoprevention, consistent with well-known compensatory increases in SAMDC activity (46). SAMDC activity did increase in DFMO treated chest and ear skin from a mean of 100 to 167 pmol/min/mg protein (data not shown).

Effect of DFMO Chemoprevention on Epidermal TUNEL Staining, Keratin Protein Expression, and Transgene Expression. A potential mechanism for the anticarcinogenic efficacy of DFMO could be activation of apoptosis. Our analysis focused on the 32-week time point because this was the transition stage to cancer, and induction of apoptosis could also underlie the marked regression of papillomas in the 28 – 32-week DFMO treatment group. Overall, TUNEL-positive cells were rarely detected in either untreated transgenic or nontransgenic mice (data not shown). DFMO treatment of transgenic mice from either 4 – 32 or 28 – 32 weeks was associated with an increase in TUNEL-positive cells in the upper granular layers of interfollicular epidermis and in the inner root sheath of hair follicles and in sebaceous glands (data not shown). However, TUNEL-positive cells were rare in the ORS or interfollicular epidermal basal cell layer, which is the cell population wherein mutations contributing to carcinogenesis would persist and accumulate, in DFMO-treated transgenic mice.
Analysis of the response of a panel of mouse keratins-14, -5, -6, and -10 and the envelope-associated protein, loricrin, to DFMO chemoprevention did not reveal a consistent difference between untreated and treated transgenic mice (data not shown). In particular, interfollicular expression of keratin-6 was detectable to a similar degree in both untreated and DFMO-treated transgenic mice. Because interfollicular keratin-6 expression is detected in hyperproliferative epidermis (47), these data are consistent with a limited effect of DFMO on keratinocyte proliferation in the interfollicular epidermis of K14-HPV16 transgenic mice. The level of transgene expression, determined by mRNA in situ hybridization was also similar in treated and untreated transgenic mice.

DISCUSSION

K14-HPV16 transgenic mice spontaneously develop epidermal cancers progressing through a series of well-defined stages associated with characteristic alterations in markers of proliferation, keratinocyte differentiation, and expression of receptor tyrosine kinases (28, 35, 45). However in the present study, many of these markers, with the exception of BrdUrd incorporation in the latter stages of neoplastic progression, were not affected by DFMO chemoprevention. Therefore, we investigated whether the model possessed additional histopathological stages of neoplastic progression and biochemical or molecular markers for studying chemoprevention. Histopathological
analysis uncovered microinvasive cancers which appeared in 40% of transgenic mice by 32 weeks of age, an incidence of malignancy that was surprisingly similar to the eventual incidence of visible cancer at 1 year of age. These data, combined with complete inhibition of microscopic malignancies by DFMO treatment from 4 to 32 weeks of age, supported the notion that 32 weeks of age could be a new endpoint for chemoprevention studies in this model. Further histopathological analysis of both the 16- and 32-week time points uncovered previously unknown neoplastic lesions of the chest and ear, hyperproliferative follicular/interfollicular chest dysplasias, and sessile, dysplastic ear papillomas. Detection of microinvasive malignancies within these lesions supported the hypothesis that they were a more advanced stage of malignancy than dysplasia. In addition, their propensity for malignant conversion also suggested that these precursor lesions could be SEBs in chemoprevention studies. Subsequently, the histopathological response of these precursors to DFMO chemoprevention from 4 to 32 weeks and therapeutic DFMO treatment from 28 to 32 weeks of age supported their utility as SEBs. Histopathological analysis also correlated with the biochemical response to chemoprevention, such that the ODC activity in the ear was completely normalized by DFMO but remained elevated in chest skin. Taken together, the distinctive precursor lesions of spontaneous carcinogenesis and the ultimate morphological and biochemical responses to DFMO chemoprevention and therapy are further support for two distinct pathways of neoplastic progression specific for either chest or ear skin (Fig. 8). As such, this study provides a paradigm for future efficacy screens of chemopreventive and therapeutic regimens having potential activity in either pathway.

Comparison of chest and ear precursor lesions of K14-HPV16 transgenic mice with neoplastic lesions developing in either classical initiation/promotion, “two-stage” chemical carcinogenesis protocols (48), or other transgenic models of epidermal carcinogenesis (reviewed in Refs. 45 and 49), identified both common and unique elements. The most frequent neoplastic lesion in the two-stage model is the pedunculated papilloma (48, 50). These chemically induced papillomas can be selected for either high or low potential of malignant conversion by manipulation of the promotion regimen (51). Papillomas are also intermediate lesions in several transgenic models of targeted expression of oncogenes (45, 52, 53) or ODC (32, 33, 44) to the epidermis. A common element of papilloma formation in these transgenic models is that most require either 7,12-dimethylbenz[a]anthracene or 12-O-tetradecanoylphorbol-13-acetate, are sessile, and are restricted in distribution to the ear. Despite differences between induction of papillomas and their appearance, a unifying feature among two-stage and transgenic models, including our own, is the exquisite sensitivity of these lesions to both chemoprevention and therapeutic regression by DFMO (16, 17, 44). In contrast to papillomas, lesions similar to hyperproliferative follicular/interfollicular chest dysplasias of K14-HPV16 transgenic mice have been documented in only a handful of other reports. “Acanthotic” lesions, which resemble the chest precursors of K14-HPV16 mice, develop on the trunk of bovine K5-v-Ha-ras transgenic mice and also progress to malignancy (49). Both HK1-HPV18 E6/E7 transgenic mice and the two-stage chemical carcinogenesis model develop neoplastic lesions that are histopathologically similar to chest precursors in our transgenic mice; yet they either do not progress to malignancy (54) or are not the immediate antecedents to invasive cancers (55).

One explanation for both the different biology of carcinogenesis in chest and ear skin and the disparate response of particular epidermal elements to DFMO in our study is the presence of distinct types of proliferating stem and transient amplifying keratinocytes as targets of neoplastic transformation (49, 56–58). Slow cycling, “label retaining,” stem cells have been shown to exist in the “bulge” region of the hair follicle ORS (59) and in the interfollicular epidermis (56). However, it is still unclear whether follicular stem cells are different from their counterparts in the interfollicular epidermis (60). When stem cells replicate, they give rise to either more stem cells or to transient amplifying cells. Transient amplifying cells are farther along the differentiation pathway such that they replicate for a limited number of divisions, then terminally differentiate (42, 61). Our study suggests that hair follicle stem cells in the chest may be targets for malignant conversion, with wounding as a possible cofactor in combination with HPV oncogene expression. Additional features of chest cancers, including a less differentiated histopathology, more aggressive biology, expression of keratin proteins found in simple epithelia, and resistance to “late stage” DFMO treatment, point to their origin from a stem cell that is more primitive compared with counterparts in the ear (49). Conversely, the biology of ear cancers, including a well-differentiated histopathology, a more benign malignant behavior, and marked sensitivity to DFMO treatment from 28 to 32 weeks of age, suggests that these malignancies are derived from progenitor cells that are less primitive than their counterparts in the chest (49). Heterogeneity of progenitor and proliferative cells within the epidermis may also underlie the fact that interfollicular hyperplasia and dysplasia were
resistant to DFMO chemoprevention or late-stage therapy, in sharp contrast to ear papillomas and the hair follicle ORS of the chest (57, 58).

The response of ODC enzyme activity and tissue polyamine content to both spontaneous carcinogenesis and DFMO chemoprevention also supported the hypothesis that carcinogenesis in the chest or ear skin of K14-HPV16 transgenic mice was different. The marked increase of ODC activity at the initial stage of neoplastic progression in ear skin, compared with the gradual elevation of enzyme activity in chest skin attaining significance only in the last stage of premalignant progression, suggested that polyamine requirements during epidermal neoplasia may be site specific. Moreover, ODC activity in the ear was more sensitive to DFMO inhibition compared with chest skin. One simple pharmacokinetic explanation is that the level of DFMO is higher in ear compared with chest skin. However, given the rapid uptake and widespread distribution of orally administered DFMO (62), this explanation is unlikely. Alternatively, more complex mechanisms involving cellular drug uptake and metabolism may underlie the differential therapeutic decrease in ODC activity at these two sites.

Our emerging understanding of the molecular response of the host cell to expression of the HPV oncoproteins also suggests one possible mechanism for induction of ODC activity and increase in polyamine content during epidermal carcinogenesis in untreated transgenic controls. E-box elements, c-Myc DNA binding sites, are found either in the 5’ regulatory region of the human (63) or in the first intron of the rodent ODC gene (64). Moreover, ODC mRNA levels increase in response to overexpression of c-Myc (63, 65). HPV E7 has been shown to upregulate c-myc expression by abrogation of transrepression by retinoblastoma protein (66). Furthermore, we have shown that the neoplastic epidermis of K14-HPV16 transgenic mice contains an increased amount of c-myc mRNA, and that keratinocytes transfected with HPV E7 predominantly contain c-Myc protein in complex with Max, the constitutively expressed protein partner of c-Myc in the heterodimer that binds to the E-box (34). As such, expression of the HPV16 E7 oncoprotein in the epidermis may, in part, enhance ODC activity by transcriptional mechanisms coordinated by c-Myc.

In summary, the response of newly discovered chest and ear pre-cursor lesions to DFMO supported their utility as SEBs in chemoprevention studies with K14-HPV16 transgenic mice. Moreover, hyperplasia and dysplasia, which are universal in 4- and 8-week-old controls, can be also used as SEBs to study the biology of other agents or regimens that may possess activity at earlier stages of neoplastic progression. Collective analysis of histopathology and BrdUrd incorporation in the present study suggests that DFMO alters fibroepithelial remodeling to prevent formation of dysplastic papillomas in the ear and produces a diminution of ORS hyperplasia and proliferation, which may limit the size and extent of hyperproliferative follicular/interfollicular chest dysplasias. Because the consequences of inhibition of ODC activity and decrease in polyamine metabolism in intact animals can be protein, the precise mechanisms of DFMO chemoprevention remain obscure. However, delineation of novel SEBs characteristic of specific stages of neoplastic progression highlights the use of K14-HPV16 transgenic mice as platforms for testing the biology of prevention regimens at multiple stages of squamous epithelial carcinogenesis.

ACKNOWLEDGMENTS

We thank Allan Balmain and Karen Smith-McCune for comments on the manuscript.

REFERENCES

Difluoromethylornithine Chemoprevention of Epidermal Carcinogenesis in K14-HPV16 Transgenic Mice

Jeffrey M. Arbeit, Rebecca R. Riley, Bing Huey, et al.


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/59/15/3610

Cited articles  This article cites 60 articles, 36 of which you can access for free at:
http://cancerres.aacrjournals.org/content/59/15/3610.full.html#ref-list-1

Citing articles  This article has been cited by 11 HighWire-hosted articles. Access the articles at:
/content/59/15/3610.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.