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Advances in Brief

Evidence That the Epidermal Targets of Carcinogen Action Are Found in the Interfollicular Epidermis or Infundibulum as well as in the Hair Follicles[1]

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Abstract

Actively cycling, transit-amplifying cells and quiescent cells including stem cells are found in the layer of the epidermis and hair follicles. To determine the origin of skin tumors, we completely removed the interfollicular epidermis of carcinogen-initiated mice by an abrasion technique known to leave the hair follicles undisturbed. The interfollicular epidermis of the abraded mice quickly regenerated from cells in the hair follicles, after which time tumor promotion was begun. Mice in which the interfollicular epidermis had been removed developed papillomas and carcinomas; however, the number of papillomas throughout 40 weeks was half that of the unabraded mice. Carcinoma responses were not significantly different in the abraded and unabraded groups. These results are consistent with the hypothesis that the targets of tumor initiation are stem cells found in the hair follicles and, to a lesser degree, in the interfollicular epidermis.

Introduction

Benign and malignant cutaneous neoplasms can be induced on the backs of mice after a subthreshold exposure to a carcinogen (initiation) and subsequent chronic regenerative epidermal hyperplasia (promotion). Initiation is thought to involve the conversion of some of the epidermal cells into latent neoplastic cells; promotion elicits expression of the neoplastic change (1–3). An important problem in skin cancer research is the identification of the targets cells for chemical and physical carcinogens (4). Conceivably, any keratinocyte capable of proliferation could become and remain initiated. However, there is mounting evidence that a subpopulation known as stem cells are the targets of the carcinogen (reviewed in Ref. 5). A number of investigations have demonstrated that the initiated cells persist in the epidermis essentially for the life of the animal (reviewed in 5), which, in view of the continual renewal of the epidermis and hair follicles, suggests that the initiated cells may not be simply any proliferative cell, but stem cells. Most evidence has placed these target cells in the hair follicles (reviewed in Ref. 6). Direct evidence that initiated cells originated from the hair follicles came from the work of Argyris (7, 8), who demonstrated that papillomas and carcinomas were promoted in the skin of initiated mice by repeated abrasions resulting in the removal of the interfollicular epidermis. Nevertheless, underlying this evidence that cutaneous neoplasms have a follicular origin are mathematical models supported by cellular kinetic data that the interfollicular epidermis also has a population of stem cells (reviewed in Ref. 9). To probe further the hypothesis that the initiated cells in two-stage carcinogenesis and hence, the stem cells, have an interfollicular as well as a follicular origin, we completely removed the interfollicular epidermis from carcinogen-exposed mice by an abrasion technique known to spare the hair follicles and subsequently promoted tumors. We hypothesized that, if the initiated cells were solely in the hair follicles, the tumor responses should be the same in control and abraded mice. Alternatively, if a subset of initiated cells resided in the interfollicular epidermis, then the tumor responses would be greater in the unabraded group than in the abraded group. In this report, we demonstrate that removal of the interfollicular epidermis reduces by half the number of papillomas, although the number of carcinomas remains the same in abraded and unabraded mice.

Materials and Methods

Animals and Husbandry. Female CD-1 female mice (VAF Plus; Charles River Laboratories, Wilmington, MA) and SENCAR mice (National Cancer Institute, Frederick, MD) were received at 6 weeks of age and were housed 5 per 86.25 inch^2 cages on Beta Chips (Northeastern Products, Warrenburg, NY) in an air-conditioned room (21°C–22°C) with a 12-h light cycle. Cages were changed twice weekly; food (Lab Chow #5020; P.M.I. Feeds, Inc., St. Louis, MO) and tap water were available *ad libitum*. The mice were clipped with electric clippers (Oster Golden A5 with a #40 blade; Oster Professional Products, McMinnville, TN) when 7 weeks of age; at which time, all mice were in the resting stage of the hair growth cycle. Sentinal mice housed in the same room remained negative toward the common murine pathogens throughout the project. All experiments involving animals were carried out with the approval of the Institutional Animal Care and Use Committee and conformed to the standards set forth by the NIH Guide for the Care and Use of Laboratory Animals.

Chemicals. DMBA[3] was purchased from Aldrich (Milwaukee, WI). It was dissolved at a concentration of 1000 nmol/ml in acetone (high-performance liquid chromatography grade; Fisher Scientific, Fair Lawn, NJ). TPA was purchased from Sigma Chemical Co. (St. Louis, MO) and was dissolved at 85 nmol/ml in acetone.

Abrasion Technique. The interfollicular epidermis was removed as described by Argyris (7). Briefly, mice were anesthetized with an injection of sodium pentobarbital before clipping and depilation with Nair (Carter-Wallace, New York, NY). After scrubbing with 70% ethanol and drying under an incandescent lamp, the basal and suprabasal layers in an area of 4 cm^2 of the interfollicular epidermis were removed by careful abrasion with a felt wheel mounted on a Dremel Moto-tool (Racine, WI). After abrasion, the skin was shiny and smooth, and there was no blood. One day later, the abraded area was covered by a fibrin crust, which fell off after 3–4 days, exposing the newly regenerated epidermis. Control mice were anesthetized, clipped, and depilated. Ten each of CD-1 and SENCAR mice were euthanized immediately after abrasion to ascertain microscopically the complete removal of the interfollicular epidermis.

Carcinogenesis Experiments. Carcinogenesis experiments for CD-1 and SENCAR mice were run for 52 weeks and were repeated for each mouse stock. The mice were treated topically one time between 9:30 and 11:00 a.m. with either 200 μl of high-performance liquid chromatography-grade acetone (Fisher Scientific Co., Fair Lawn, NJ) or with 200 nmol of DMBA in 200 μl of acetone (CD-1 mice). SENCAR mice were initiated with 10 nmol of DMBA. One week later, groups of mice were abraded as described above. Four weeks after abrasion, twice weekly tumor promotion with either 200 μl of acetone or

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[3] The abbreviations used are: DMBA, 7,12-dimethylbenz[*a*]anthracene; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

with 17 nmol of TPA (CD-1 mice) or 3 nmol (SENCAR mice) in 200 μl of acetone was begun and was continued for 20 weeks. The specific details of the experimental groups for the carcinogenesis experiments are shown in Table 1. The mice were observed weekly for the presence of skin lesions. Papillomas were counted as such when >1 mm in diameter; suspected cutaneous malignancies were identified by their broad base, elevated margin, and intracutaneous infiltration, were verified at autopsy, and were confirmed histopathologically. Mice with skin lesions >1 cm in diameter or appearing stressed in any way were removed from the experiment and were euthanized.

Statistical Analysis. Statistical analyses were performed with the Student's *t* test and the Lifetable Analysis.

Results

Fig. 1*A* shows the skin of a typical CD-1 mouse. There were no obvious morphological differences between CD-1 and SENCAR mice. Fig. 1*B* demonstrates the complete removal of the interfollicular epidermis after felt-wheel abrasion. The abrasion procedure does not cause obvious damage to the hair follicles. The kinetics of regeneration in both CD-1 and SENCAR mice were investigated both morphologically and by counts of epidermal nuclei and were found to be similar to those reported previously (10).

When control mice were clipped and depilated but not abraded 1 week after initiation with DMBA, the first papules were seen at week 5 of promotion in both the CD-1 and the SENCAR mice. In abraded mice of both stocks, the first papules were noted at week 4. The maximal papilloma responses of unabraded mice were observed at week 18 for CD-1 and SENCAR mice (Fig. 2, *A* and *B*). The papilloma responses of abraded CD-1 and SENCAR mice were significantly less than those of the unabraded mice ($P < 0.001$ for all experiments) from weeks 10 to 40. Numbers of mice bearing papillomas approached 100% in both the abraded and unabraded groups (Fig. 2, *C* and *D*). Of the control groups, the number of papillomas per mouse at week 18 was 0 ± 0 (SD) for the acetone/unabraded/acetone, acetone/abraded/acetone, and DMBA/unabraded/acetone. The number of papillomas at week 18 was 0.1 ± 0.1 for the acetone/unabraded/TPA and for the DMBA/abraded/acetone groups and was 0 ± 0.1 for the acetone/abraded/TPA group. The controls of the carcinogenesis experiments described above demonstrated that removal of the epidermis by a single abrasion acted neither as a complete carcinogen, an initiator, nor as a promoter of two-stage carcinogenesis under the conditions used.

As shown in Table 2, the first suspected carcinomas appeared in both CD-1 and SENCAR mice at approximately the same time, whether or not the mice had been abraded. For both CD-1 and SENCAR mice, there was no statistical support for a difference in the number of carcinomas at weeks 35, 45, or 52. With the exception of two mice having two carcinomas, all carcinomas occurred singly. One of the two mice was in a DMBA/unabraded/TPA group, and the other mouse was in a DMBA/abraded/TPA group.

Table 1 *Protocols and treatment groups to determine the effects of a single abrasion on tumor promotion*

Protocol	No. of mice	First treatment (initiation)[a]	Second treatment[b]	Third treatment (promotion)[c]
1	10	Acetone	Unabraded	Acetone
2	10	Acetone	Abraded	Acetone
3	40	Acetone	Unabraded	TPA
4	40	Acetone	Abraded	TPA
5	10	DMBA	Unabraded	Acetone
6	40	DMBA	Abraded	Acetone
7	40	DMBA	Unabraded	TPA
8	40	DMBA	Abraded	TPA

[a] CD-1 female mice were treated with a single topical application of 200 nmol of DMBA in 200 μl of acetone. SENCAR female mice were treated with 10 nmol of DMBA.
[b] One week after DMBA treatment, the interfollicular epidermis was removed by abrasion with a felt wheel.
[c] Twice weekly tumor promotion with either 200 μl of acetone or TPA (17 nmol for CD-1 mice, 3 nmol for SENCAR mice) in 200 μl of acetone was begun 4 weeks after abrasion and was continued for 20 weeks. Carcinomas developed until 52 weeks. Animals remained in the experiment until they developed cutaneous lesions of 1 cm^2.

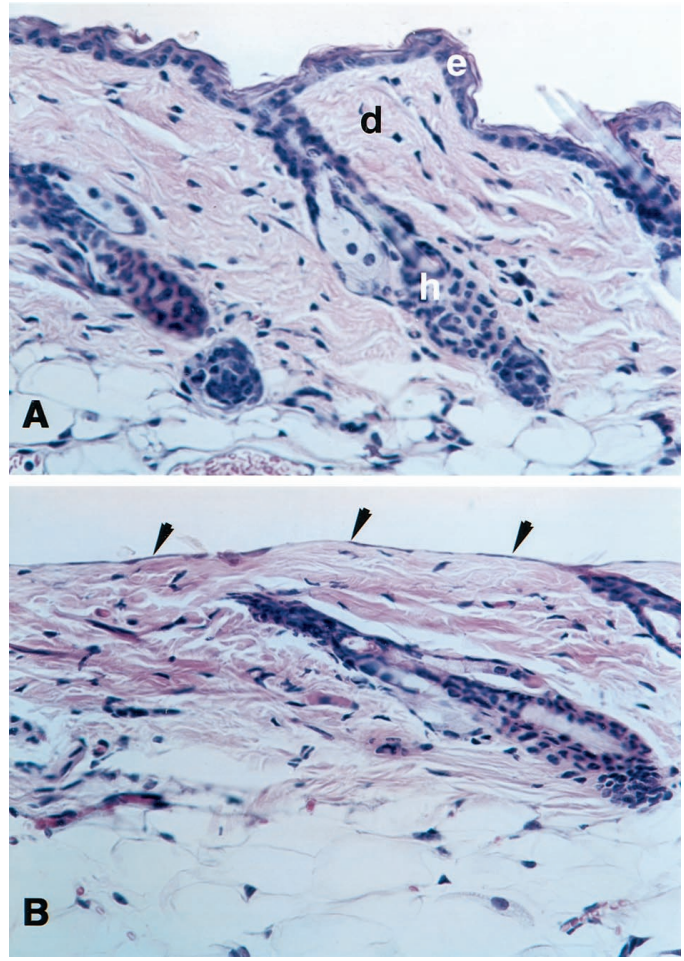

Fig. 1. Photomicrographs of skin from CD-1 female mice. *A*, unabraded skin showing the epidermis (*e*), dermis (*d*), and hair follicle (*h*). *B*, skin from an abraded mouse showing the complete removal of the epidermis (*arrowheads*). Note that the hair follicle remains undisturbed. *A* and *B*, ×400, hematoxylin and phloxine B.

Discussion

We have demonstrated that when the interfollicular epidermis of initiated mice was removed one time by abrasion, the mice developed papillomas and carcinomas upon subsequent tumor promotion. However, the number of papillomas in all of the abraded groups was clearly less than the papilloma number in the unabraded groups and remained reduced through 40 weeks. In contrast, the number of carcinomas was essentially the same in abraded and unabraded mice.

Our results confirm earlier observations suggesting that the hair follicles were the site of the target cells. We have shown that the carcinogen-initiated cells in the cutaneous epithelium not only are persistent but are also quiescent and resistant to treatment with doses of 5-fluorouracil that cause epidermal sloughing in approximately half of the mice (11). Hansen and Tennant (12, 13) reported that cutaneous papillomas induced by TPA in the TG:AC transgenic mice were derived from individual hair follicles in which the mutated v-Ha-*ras* transgene was expressed. However, it is not clear that carcinogenesis in this transgenic mouse faithfully reproduces the multistage model of cutaneous carcinogenesis. In

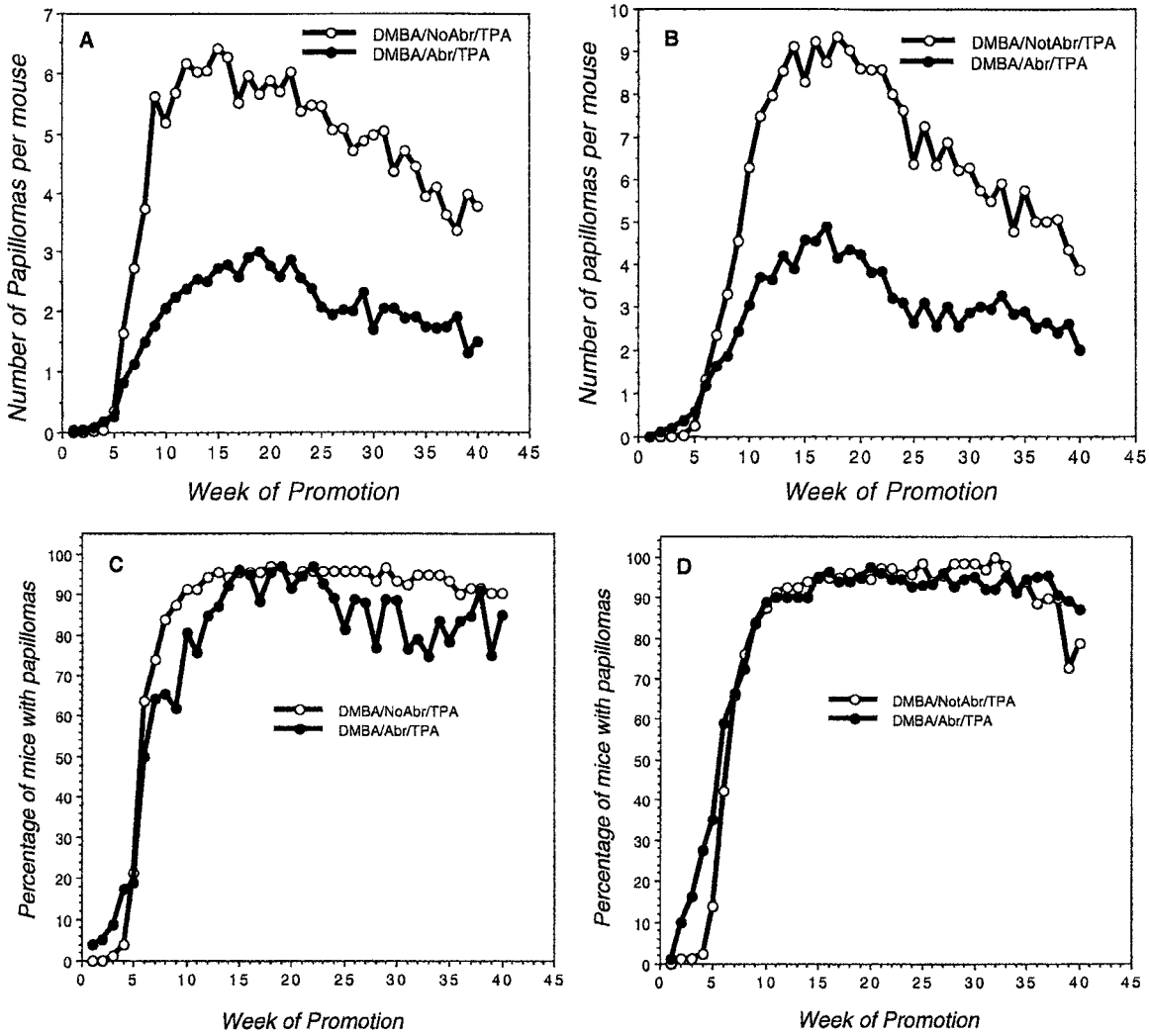

Fig. 2. Effects on the papilloma responses of removal of the interfollicular epidermis by a single abrasion 1 week after tumor initiation with DMBA and prior to tumor promotion with twice weekly application of TPA for 20 weeks. *A* and *C*, SENCAR mice. *B* and *D*, CD-1 mice. Graphs represent the average responses in two such experiments as outlined in Table 1. Note that abrasion reduced by half the papilloma responses in both mouse stocks.

contrast, Binder *et al.* (6) found direct association of interfollicular epidermis together with hair follicles in early precursor lesions of papillomas, raising the question of whether lesions could have had an interfollicular origin.

Table 2 *Effects of removal of the interfollicular epidermis by abrasion on cutaneous malignancies in CD-1 and SENCAR female mice*

Experiment[a]	First carcinoma week[b]	Percentage of mice with carcinoma[c]		
		Week 35	Week 45	Week 52
CD-1 Not abraded	24	10/28 (35.7%)	22/27 (81.5%)	25/26 (96.2%)
CD-1 Abraded	25	6/26 (23.1%)	18/22 (81.8%)	20/22 (90.9%)
SENCAR Not abraded	24	11/22 (50.0%)	18/22 (81.8%)	18/22 (81.8%)
SENCAR Abraded	24	12/26 (46.2%)	20/24 (83.3%)	21/24 (87.5%)

[a] Forty mice in each group were either clipped and depilated or abraded as outlined in Table 1.

[b] Suspected carcinomas were identified by their broad base, elevated margin, and intracutaneous infiltration. They were verified at autopsy and were confirmed histopathologically. The fraction is the cumulative number of mice with carcinoma/number of mice at risk. The value in parentheses is the fraction expressed as a percentage. During the experiments, mice were euthanized if they became ill or developed any lesion >1 cm^2.

[c] Statistical analysis was performed by a Lifetable Analysis. There was no statistical support for differences between experimental groups at any observation time.

Our results support the hypothesis that the target cells for carcinomas and for many papillomas reside in the hair follicles but that target cells for some papillomas are present in the interfollicular epidermis or in the upper, infundulum of the hair follicles. Whether the target cells for all possible carcinomas reside in the hair follicles is an important question that is not conclusively answered in this study because the carcinomas occurred, for the most part, singly in abraded and unabraded mice. We have demonstrated that the cells in the upper, infundibular portion of the follicle contribute to the re-epithelialization of the dermis after abrasion and that these cells are quickly lost from the epidermis once re-epithelialization is completed (10). That the carcinoma responses were the same in abraded and unabraded mice support the hypothesis that stem cells with the greatest proliferative potential are found in the hair follicles.

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