Protein Kinase C-ε Transgenic Mice: A Unique Model for Metastatic Squamous Cell Carcinoma

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Abstract

Squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) are the most common forms of human skin cancer. BCC is slow growing and mostly localized, whereas SCC metastasizes to the regional lymph nodes and subsequently to distal organs. In murine skin carcinogenesis models for SCC, the incidence of metastasis is very low. We report here that FVB/N transgenic mice, which overexpress (−18-fold) epitope-tagged protein kinase C-ε (T7-PKCε) protein in the epidermis provide a unique murine model system for highly malignant/metastatic SCC. Skin tumors were developed by the initiation-promotion protocol (initiation with 100 nmol 7,12-dimethyl-benz[a]anthracene; promotion with 5 nmol 12-O-tetradecanoylphorbol-13-acetate twice weekly). T7-PKCε transgenic mice showed 92% suppression of papilloma development compared with wild-type littermates after 23 weeks of tumor promotion. However, within 15–20 weeks of 12-O-tetradecanoylphorbol-13-acetate promotion, 40% of T7-PKCε mice developed at least one carcinoma compared with 7% of the wild-type mice. All carcinomas from T7-PKCε mice appeared without prior papilloma formation. Interestingly, 7,12-dimethyl-benz[a]anthracene alone resulted in the development of squamous cell carcinomas in 22% of T7-PKCε mice, whereas wild-type littermates developed no tumors. Histopathological analysis of tumors from multiple T7-PKCε mice revealed moderately differentiated SCC invading the dermal region with neoplasia appearing to originate and invade from the hair follicle. Carcinomas of T7-PKCε mice rapidly metastasized to regional lymph nodes within 3 weeks of appearance. In wild-type mice, the grade of the invading tumors, originating from interfollicular epidermis, was pathologically categorized as well-differentiated SCC and remained localized to the dermis. The T7-PKCε transgenic mice may provide a rapid and unique in vivo model to investigate metastatic SCC.

Introduction

The majority of human cancers originate from epithelial tissue (1). A common cancer of epithelial origin is NMSC with >700,000 new cases diagnosed each year in the United States (2). NMSC includes both BCC and SCC. Human cutaneous SCC metastasizes at a rate of 2–6% over the span of several years after initial diagnosis (3). The highly malignant form invades and destroys tissue, and then metastasizes initially to the regional lymph node before distal organs are affected (3). Animal models have been important for studying the mechanisms of tumor development and progression, and murine skin model systems are still essential contributors to our understanding of the multistep nature of chemically induced carcinogenesis (4). The predominant induced NMSC of mice is SCC. Although SCC of mouse skin invades the dermal region, the incidence of malignant metastatic conversion is low and requires a long latency period of ∼1 year (5, 6). The murine skin model system used at present lacks the capability to study metastatic development in a timely manner (7–9).

To define the distinct role of individual PKC isoforms in the signal transduction pathways resulting in mouse skin tumor promotion by TPA, we reported generation of FVB/N transgenic mice expressing an T7-epitope-tagged PKCα, PKCδ, or PKCe under the control of the human keratin 14 promoter/enhancer (10–12). Transgenic expression of T7-PKCα did not affect tumor promotion susceptibility. The T7-PKCδ- and T7-PKCε-expressing transgenic mice exhibited different sensitivities to the induction of mouse skin tumors by initiation with DMBA and twice-weekly promotion with TPA. The increased expression of T7-PKCδ protein in the epidermis (∼8-fold) suppressed the formation of both skin papillomas and carcinomas by 70% (11). In contrast, the increased expression of T7-PKCε protein in the epidermis (∼18-fold) almost completely suppressed papilloma formation but still resulted in the development of SCC (12).

Here we present evidence consistent with the hypothesis that T7-PKCε papilloma-independent carcinomas are pathologically distinguishable, based on histogenesis, from wild-type mouse tumors. The invading T7-PKCε tumors were pathologically categorized as MDSC, which rapidly metastasized to regional lymph nodes. In contrast, malignant tumors from wild-type mice, pathologically categorized as WDSC, invaded the dermis and s.c. tissues, but remain localized. In T7-PKCε mice, the tumors appeared to originate from the hair follicle within squamous cells located near the sebaceous gland ("bulge region"). The bulge cells are postulated to be progenitor or stem cells for the hair follicle and epidermis (13, 14). In contrast, WDSC derived from papillomas in wild-type mice appeared to largely originate from the interfollicular epidermis. The T7-PKCε transgenic mouse provides a unique opportunity to study the origin and events necessary for malignant progression by using this new model of metastatic SCC.

Materials and Methods

Materials. TPA was purchased from Alexis Corporation (San Diego, CA). DMBA was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). FVB/N mice, 7–9 weeks of age, were purchased from Taconic (German-town, NY).

Mice. The generation of mice for the tumor promotion experiments was performed by mating heterozygous F5 males with wild-type FVB/N mice. The transgene was detected by dot blot analyses of genomic DNA from tail biopsies using the radiolabeled EcoRV/BamHI fragment from pGEM3Z-K14 β-globin vector, which encompasses ∼1 kb of the K14 promoter and the entire β-globin intron.

Histology. The tissue to be examined was excised promptly after euthanasia and immediately placed in 10% neutral-buffered formalin. Normal tissue was fixed for 1 h in formalin and then embedded in paraffin. Carcinomas and lymph nodes required fixation times of 2–3 h. Sections (4-μm thick) were cut
for H&E staining. Carcinomas were examined by a pathologist (T. D. O.). Previous studies demonstrated concordance between gross classification of skin tumors (papilloma versus carcinoma) and subsequent microscopic evaluation by a pathologist (T. D. O.).

**Tumor Induction Experiments.** For mouse skin tumor initiation, a single 100-nmol dose of DMBA in 0.2 ml of acetone was applied topically to the shaved backs of 7–9-week-old female mice. Two weeks after initiation, TPA (5 nmol) in 0.2 ml of acetone or acetone alone was applied twice weekly to the dorsal skin for the duration of the experiment. Tumor incidence and multiplicity were observed weekly starting at 8 weeks of TPA promotion. The number of mice for each group was as follows: DMBA + TPA, 15 wild-type mice; DMBA + TPA, 15 T7-PKCε mice; DMBA + aceton, 14 wild-type mice, 15 T7-PKCε mice. Carcinomas were recorded by gross observation as downward-invading lesions. Carcinoma-bearing mice were observed for abnormal tumor growth in the lymph nodes.

**Statistical Analysis.** Analyses were performed using the MSTAT computer program, provided by Dr. Norman Drinkwater, University of Wisconsin, Madison, WI. Two-sided P values were calculated for tumor and metastasis multiplicity by Wilcoxon’s rank-sum test. Two-sided P values comparing tumor incidence were calculated using Fisher’s exact test.

**Results**

**Carcinoma Development and Metastatic Malignant Progression in T7-PKCε Transgenic Mice.** The T7-PKCε mouse line 215, which expressed T7-PKCε protein at concentrations ~18-fold higher than endogenous PKCε levels (12), was further evaluated for the development of carcinomas by the DMBA initiation and TPA tumor promotion protocol. In this experiment, female wild-type and T7-PKCε transgenic mice were treated topically with 100 nmol of DMBA in 0.2 ml of acetone. Two weeks later, 0.2 ml of aceton or 5 nmol of TPA in 0.2 ml of acetone was applied twice weekly to the dorsal skin. At the beginning of the experiment, the 7–9-week-old mice exhibited no phenotypic abnormalities. Treatment with TPA for 23 weeks elicited an average of 12 papillomas/wild-type mouse (Table 1). However, in accordance with our previous findings (12), the T7-PKCε mice averaged <1 papilloma/T7-PKCε mouse (Table 1). The papillomas that developed in T7-PKCε mice were also much smaller than wild-type papillomas (data not shown). Despite the low papilloma burden, the T7-PKCε mice developed carcinomas independently of papilloma development (Fig. 1B). After 23 weeks of tumor promotion, 6 of 15 (40%) transgenic mice were evaluated by gross examination as having at least one carcinoma, compared with 1 of 15 (7%) of the wild-type mice (Table 1 and Fig. 1B). Wild-type carcinomas developed from existing papillomas. Additionally, 3 of 15 transgenic mice treated with DMBA + aceton alone also developed papilloma-independent carcinomas (Table 1 and Fig. 1A). Wild-type mice treated with DMBA + aceton developed no papillomas (Table 1 and Fig. 1A). Because the treatment parameters were identical between the current experiment and our previously reported experiment (12), we normalized and combined the experimental data to determine whether the development of carcinomas in T7-PKCε mice after DMBA initiation alone was statistically significant. From the combined data, we determined that DMBA + aceton treatment elicited carcinoma development in 7 of 31 (22%) T7-PKCε mice, whereas wild-type mice never developed carcinomas (Table 2). From the analysis of the combined data, we conclude that DMBA + acetone is sufficient to induce carcinoma development in T7-PKCε mice (Table 2).

T7-PKCε mice rapidly developed tumors in regional lymph nodes within 3 weeks after positive identification of carcinomas by gross observation (Fig. 1B). Three of six mice positive for carcinomas contained regional lymph nodes that bore tumors (Table 1). The T7-PKCε transgenic mice that developed carcinomas with DMBA + aceton treatment did not have evidence of enlarged lymph nodes (Fig. 1B). However, the positive identification of the carcinomas was less than 3 weeks before the mice were sacrificed at the conclusion of the experiment.

**Primary Tumors Arising in T7-PKCε Mice Are Derived from the Hair Follicle.** Wild-type and T7-PKCε mice that were positive for carcinoma formation were sacrificed 1 week after the last treatment with TPA or acetone. The carcinoma and surrounding uninvolved skin were removed, fixed, and embedded. Regional lymph nodes with evidence of tumor growth by gross observation were also isolated, along with apparently normal lymph node in the same animal.

By gross observation, both wild-type mouse and T7-PKCε mouse carcinomas were identified by dark red color or the presence of blood clot on the skin surface. As the lesions progressed, necrosis occurred on the surface of the cancer, and surface ulceration resulted. Microscopically, cancer cells were identified by the presence of large pleomorphic nuclei with prominent nucleoli and frequent mitoses. Areas of intracellular keratinization and focal extracellular keratin deposits were identified. The cell cytoplasm was abundant, and the cell surface exhibited intercellular bridges. Neutrophils were identified focally adjacent to keratin pearls. The tumors from T7-PKCε transgenic mice were moderately MDSC based on a small number of focal areas with typical squamous epithelium or keratin formation and a large number of areas composed of largely undifferentiated cells. In histological sections of MDSC from two T7-PKCε mice initiated with DMBA and treated for 23 weeks with 5 nmol of TPA, malignant cells were seen streaming from the hair follicle, often in the region of the sebaceous gland (Fig. 1, C–E). This process often involved multiple adjacent hair follicles.

DMBA-initiated mice that had been treated for only 8 weeks with TPA or acetone were also harvested to determine the origin of premalignant lesions. After 8 weeks of TPA treatment, T7-PKCε mice displayed focal areas of increased hair follicle width, epidermal hyperplasia, and hyperkeratosis. Possible premalignant lesions were identified arising from hair follicles; these lesions had cells with enlarged nuclei and prominent nucleoli and showed outward expansion from the hair follicle (Fig. 2).

Multiple T7-PKCε transgenic mice exhibited enlarged regional lymph nodes after the identification of primary tumor (Fig. 1B). The lymphoid tissue, identified by the presence of numerous collections of well-differentiated lymphocytic cells, was infiltrated by SCC (Fig.

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**Table 1** Metastatic SCC progression in T7-PKCε transgenic mice

Mouse skin was initiated with a single application of DMBA (100 nmol) followed by twice-weekly application of TPA (5 nmol) for 23 weeks. The changes in metastatic development and papilloma development were compared between wild-type and T7-PKCε mice.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Treatment</th>
<th>No. of mice with carcinoma</th>
<th>No. of mice with metastasis</th>
<th>Papillomas/Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>DMBA + aceton (n = 14)*</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>DMBA + TPA (n = 15)</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>T7-PKCε</td>
<td>DMBA + aceton (n = 15)</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>T7-PKCε</td>
<td>DMBA + TPA (n = 15)</td>
<td>6</td>
<td>3*</td>
<td></td>
</tr>
</tbody>
</table>

* Number of mice per group.  
* Two-sided P < 0.05 for comparison of metastatic development between wild-type and T7-PKCε mice.  
* Two-sided P < 0.001 for comparison of papilloma development between wild-type and T7-PKCε mice.
The carcinoma cells ranged in appearance from undifferentiated clusters of epithelial cells to well-differentiated squamous cells producing keratin ("keratin pearls"). Other areas showed undifferentiated carcinoma cells with numerous mitoses identified. The cell morphology was identical to that seen in the primary cancer. Focal areas in the carcinoma show extensive keratin pearl formation. G, histopathology of WDSC from a wild-type mouse that arose from a papilloma. The tumor appears to invade the dermal region from the epidermis. Note the keratin pearl. H, higher magnification of the invasive squamous tumor cells. E, epidermis; HF, hair follicle; K, keratin; Ly, lymph node; S, sebaceous gland. Magnification: C, F, and G, ×200; D, E, and H, ×400.

Discussion

T7-PKCε-expressing transgenic mice display almost no papilloma development during treatment with the two-stage DMBA+TPA tumor promotion protocol compared with wild-type littermates. However, carcinoma development appears to be enhanced compared with wild-type mice (12). In an effort to better understand the origin and development of papilloma-independent carcinomas, T7-PKCε mice on mouse skin, including epidermal hyperplasia and keratinization, should mostly have subsided. This was the case with the uninvolved skin of wild-type mice, which displayed no abnormalities (data not shown). However, the uninvolved skin of T7-PKCε mice 1 week after TPA treatment still exhibited hyperplasia of all epidermal cell layers with minimal hyperkeratosis, and small isolated foci of lymphocytic infiltrates were identified within the dermis (data not shown).
were further evaluated for the development of carcinomas by the DMBA+TPA tumor promotion protocol. We infer three conclusions from the most recent work. The first two conclusions are that papilloma-independent carcinomas, which develop in T7-PKCε mice, arise from the hair follicle and have increased metastatic potential. The third result was determined after combining our previous tumor promotion results (12) with our most recent experiments. Using statistical analysis, we were able to conclude that induction by DMBA was sufficient for SCC development in T7-PKCε mice.

Histopathological analysis of multiple T7-PKCε mice indicated that SCC of T7-PKCε mice invaded the dermal region from the hair follicle. The SCC of T7-PKCε mice rapidly metastasized to regional lymph nodes as soon as 3 weeks after positive identification of carcinoma by gross observation. The tumors from T7-PKCε transgenic mice were classified as MDSC. By comparison, the carcinomas of wild-type mice, which appeared to originate from the interfollicular epidermis of papillomas, were classified as WDSC. WDSC derived from papillomas invaded the dermal area with no evidence of metastatic progression.

The difference in metastatic potential and the different origin of malignancy provided support for the original hypothesis that T7-PKCε papilloma-independent carcinomas were pathologically distinct from wild-type mouse carcinomas. Although the papilloma-independent carcinomas appeared to originate from the hair follicle, it was possible that the origin of the tumor was not within the hair follicle. The hair follicle might have been the easiest pathway for invasion. However, this did not appear to be the case because we observed neoplastic cells arising only from the hair follicle and not the epidermis. By harvesting T7-PKCε and wild-type mice after 8 weeks of DMBA+TPA or DMBA+acetone treatments, we identified possible premalignant areas in T7-PKCε mice as early as 8 weeks after DMBA+TPA treatment. The premalignant lesions originated within the hair follicle.

The metastatic potential of a transformed keratinocyte appeared to inversely correlate with the differentiation potential of that keratinocyte in the limited number of tumors studied to date. This conclusion was based on the location of invasion and pathological categorization of T7-PKCε mouse carcinoma compared with wild-type mouse carcinoma. Bulge keratinocytes are located near the sebaceous gland within the hair follicle. Evidence suggests that these cells appear to be the stem or progenitor cells for both the hair follicle and epidermis and, therefore, would be in a less-differentiated state than epidermal cells (13, 14). These properties may increase the metastatic potential of these cells. The carcinomas of T7-PKCε mice that led to metastases were also less differentiated than carcinomas from wild-type mice. Although this study was far from conclusive, evidence suggested that malignant cells that invaded from the hair follicle were less differentiated and had a higher metastatic potential than cells that invaded from the epidermis.

Expression of T7-PKCε has the ability to induce contradictory effects in response to mouse skin tumor promotion (papilloma suppression with carcinoma induction). Because label-retaining cells located in the bulge region of the hair follicle gave rise to either a hair follicle or epidermis, the bulge cells were concluded to be bipotent stem cells (14). It is possible that T7-PKCε overexpression has a different outcome based on whether the transgene is expressed in epidermal or follicular cells. This could be one reason that T7-PKCε expression from multiple transgenic lines consistently inhibits papilloma formation but not carcinoma development. The multistep nature of carcinoma development would also suggest that elevated PKCε levels may be able to alter the regulation of several different genes to mimic the multiple molecular alterations known to occur during carcinoma development. Transgenic expression of T7-PKCε in the skin may alter the cell cycle kinetics of stem and progenitor cells. The possibility is highly unlikely that the tumor responses in the T7-PKCε-expressing mice are actually not the product T7-PKCε expression but the result of a disruption of a gene by transgenic insertion into the chromosome. In this context, it is noteworthy that a second, lower-expressing T7-PKCε transgenic mouse line (line 224), which expresses 6-fold increased PKCε in the epidermis, displays the same papilloma suppression with carcinoma induction as the higher-expressing T7-PKCε line (line 215) used in these experiments.

Several protocols are used to develop mouse skin tumors. The initiation-promotion protocol, which involves mouse skin initiation with DMBA and promotion with TPA, results in the development of...
mostly benign papillomas. More than 90% of papillomas regress after TPA treatment is stopped (15), and only a small percentage of papillomas do progress to invasive SCC (15). The initiation-promotion protocol has been further modified to enhance the conversion of skin papillomas to carcinomas, but metastatic potential is not increased (16–19).

In the mouse, present attempts to model metastasis in vivo use several experimental procedures (6). This is necessary because metastatic development within mice is rare and requires a long latency period of 1 year (5). However, the assays incompletely measure the metastatic capability of cancer cells. A classical assay involves the injection of cells into the tail vein of either immunocompromised or syngeneic mice. This assay models the latter stages of metastasis. However, the tail vein injection assay cannot be used to study the earlier invasive and angiogenic stages of malignant progression. In the use of immunocompromised mice, the importance of the immune system in metastatic progression cannot be analyzed. Subcutaneous injection of tumor cells into immunocompromised or syngeneic mice more readily evaluates the ability of tumors to invade, intravasate into the circular system, extravasate out of the circular system, invade the de novo organ site, and induce angiogenesis. However, these model systems do not allow for examination of the genesis of the cancer and ignore the complex interactions between tumor and host, especially at the tissue site where the carcinoma originated.

SCC and BCC are the most common forms of human skin cancer (2). BCC is rarely life threatening because it is slow growing and is mostly localized. SCC, unlike BCC, invades the nearby tissues (3). The first site of metastasis usually is a regional lymph node before metastatic growth in distant sites such as the lung and brain. SCC is commonly encountered in a number of epithelial tissues, including the oral cavity, esophagus, larynx, bronchi, intestines, colon, genital tract, and skin (2, 3). Effective management of SCC should include reliable biomarkers (20) for early detection of SCC and rational designed drugs for its prevention and treatment. The T7-PKCε transgenic mouse model for metastatic SCC may have selective advantages over the in vivo mouse models used at present. The carcinogen is applied topically. The procedure is convenient and inexpensive, and carcinomas, which can be monitored over time, appear rapidly within 15–25 weeks. This model could also be ideal for screening agents that may prevent the induction of metastatic SCC. This model also may be used in investigating the genetics and progression of SCC and the molecular events associated with progression and metastasis. The T7-PKCε transgenic mouse model for metastatic SCC may be a tool to achieve these goals.

References