Terminal Differentiation-resistant Epidermal Cells in Mice Undergoing Two-Stage Carcinogenesis

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ABSTRACT

We have used an in vivo-in vitro approach to investigate the cellular aspects of two-stage skin carcinogenesis. Female SENCAR mice initiated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) were promoted twice weekly with 12-O-tetradecanoylphorbol-13-acetate (TPA). Epidermal cultures from untreated or TPA-treated mice had few focus-forming cells resistant to calcium-induced terminal differentiation. Cultures from mice treated with MNNG alone formed numerous foci. Brief promotion (four TPA treatments) of MNNG-treated mice produced fewer but statistically larger foci, suggesting that TPA was selecting against more slowly growing cells. MNNG plus TPA-treated mice with very early papillomas produced more and larger foci than those due to MNNG treatment alone, suggesting that the papillomas may have comprised calcium-resistant cells. These cells may indeed be initiated cells since a permanent cell line arising after MNNG plus brief TPA treatment eventually formed histological papillomas in vivo. If calcium-resistant cells are initiated, then there were many more initiated cells in the skin (with or without TPA treatment) than papillomas expected, implying that either some initiated cells never formed papillomas, or that a significant accumulation of initiated cells had already occurred in the skin in the 2 weeks of MNNG treatment. Subsequent TPA promotion of these cells apparently produced a toxic response that passively selected for more rapidly growing initiated cells, which eventually accumulated into papillomas.

INTRODUCTION

For many years, skin cancer in mice has been the classic experimental system for chemical carcinogenesis. The sustained interest is primarily due to the disease's multistage and progressive nature (1), each stage now the subject of intensive study. The first stage, "initiation," is essentially irreversible (2-4) and is thought to involve some kind of genetic damage (5). In contrast, classical "promotion" is a reversible process occurring after initiation and requiring multiple treatments with a skin tumor promoter such as TPA.3

We are interested in the early development of initiated cells in the skin during carcinogenesis. The only in vivo assay for these cells is the subsequent appearance of tumors; however, this tells us little about preceding events. An assay that would detect initiated cells prior to tumor formation would permit us to study early events in carcinogenesis.

Recently, an in vitro assay for carcinogen-altered epidermal cells has been described, based upon the ability of such cells to resist calcium-induced terminal differentiation. Normal mouse epidermal cells grown at 37°C proliferate well in culture media with low levels of calcium (around 0.05 mM), but stop dividing

and terminally differentiate in media with standard calcium levels (approximately 1.2 mM) (6). Following carcinogen treatment, some cells continue to proliferate in standard calcium forming foci that can be scored (7). It has been suggested that these terminal differentiation- or calcium-resistant cells, since they also arise in initiated skin, may be initiated cells (8). Their number appears to be quantitatively related to initiating ability of various skin carcinogens (9, 10), and with time in culture, permanent lines of these cells become tumorigenic (11).

We have used resistance to calcium-induced terminal differentiation in vitro as an assay to detect carcinogen-altered, perhaps initiated, cells in the epidermis of adult mice undergoing two-stage carcinogenesis. Our data suggest that Ca-resistant cells may indeed be initiated cells, and that the tumor promoter TPA apparently selects for the more rapidly growing of these cells by inhibiting more slowly growing cells.

MATERIALS AND METHODS

Culture Medium. FBS (Armour Pharmaceutical Co., Kankakee, IL) was Chelex-treated (12) before use to reduce calcium content. LoCa MEM was based upon the low calcium medium used by Yuspa and Morgan (8). Eagle’s Minimum Essential Medium prepared without calcium (Cat. no. 06-174D; M. A. Bioproducts, Walkersville, MD) was supplemented with 8% Chelex-treated FBS, 5 ng/ml epidermal growth factor (Collaborative Research, Lexington, MA), 10 µg/ml transferrin (Sigma Chemical Co., St. Louis, MO), 5 µg/ml insulin (Sigma), 1 µM hydrocortisone (Sigma), 10 µM phosphoethanolamine (Sigma), 10 µM ethanolamine (Sigma), and 50 µg/ml gentamicin sulfate (Sigma). The above additives (except gentamicin) were suggested, although for a different culture medium, by Stuart H. Yuspa (National Cancer Institute, Bethesda, MD) to improve the proliferation of adult mouse epidermal cells. The final concentration of glutamate (Grand Island Biological Co., Grand Island, NY) in the medium was 3 mM. LoCa MEM prepared in this manner had an ionized calcium level of 0.05-0.06 mM (analysis provided by H. A. Fritsche, M. D. Anderson Hospital and Tumor Institute, Houston, TX). HCa MEM was supplemented with calcium chloride to raise the calcium concentration to approximately 0.6 mM.

Animal Treatment. The dorsal skin of female SENCAR mice (Frederick Cancer Research Facility, Frederick, MD) in the resting stage of the hair cycle (7-9 weeks of age) was shaved and then initiated a few days later with 4 µmol/mouse MNNG (Sigma) in 0.2 ml acetone. Promotion with 2 µg/mouse TPA (LC Services Corp., Woburn, MA), given twice weekly, was begun 1 week later. Controls for both chemical treatments received acetone only. At various times during promotion, treatment was stopped on several mice (usually three to four) from each group. These mice were set aside for cell preparation 1 week later. One such cell preparation was made from each group of mice at each time point. The experiment was terminated after 10 weeks of promotion when about half of the MNNG plus TPA-treated mice had papillomas that were readily identifiable, yet small enough (nonpapilunous, 1-mm diameter or less) to be prepared with the intact skin by the techniques described below. To corroborate any interesting responses of the papilloma-bearing skins, a second preparation of cells from each group of mice was made 2 weeks after promotion ended. All remaining MNNG plus TPA-treated mice had papillomas at that time. No papillomas were present in the other groups.

Cell Culture. Mice were killed by cervical dislocation, shaved, and washed twice with Prepydine Solution (Amsco, Medical Products Div.,
ERM, PA) and several times with water. After two more 70% ethanol washes, the dorsal skins were removed and excess fat scraped away with a rounded scalpel. The skins were then cut into manageable pieces (about 2 cm across) and placed dermis side down on dry Petri dishes to permit slight drying. Several minutes later, the pieces of skin were floated dermis side down in Petri dishes on a trypsin (Grand Island Biological Co.) solution [0.25 g/100 ml PBS (M. A. Bioproducts)] for 1 h at 31°C, then transferred to room temperature for 2 h.

After trypsinization, the epidermis was scraped from the dermis into several milliliters of culture medium containing 8% FBS to inhibit trypsin activity. The epidermal fragments were then minced with scissors and slowly stirred at room temperature for 30 min (about 30 ml total volume). The resultant cell suspension was filtered through nylon mesh to remove large fragments and then centrifuged at 200 x g for 7 min. The pellet was resuspended in LoCa MEM and 5 ml was layered over 15 ml of 45% Percoll (Sigma) (maximum 2.0 x 10^7 cells/50-ml tube). This was centrifuged at 450 x g for 15 min to remove debris (previous experiments indicated that very few viable cells remained suspended). Cells in the lower half of the Percoll phase were collected, brought to 50 ml with PBS, and centrifuged at 200 x g for 7 min.

The cells were then resuspended in LoCa MEM, counted with trypan blue (0.5 mg/ml) (Matheson, Coleman & Bell Manufacturing Chemists, Norwood, OH) for viability as reflected by dye exclusion, and plated at 1.0 x 10^6 cells in 3 ml medium/25-cm^2 collagen/fibronectin/albumin-coated (prepared as in (9), but with a MEM-based coating solution) culture flask. After two days of incubation at 31°C, the cultures were switched to HiCa MEM and incubated at 37°C for 1 month with medium changes (4 ml/flask) twice a week to select for cells resistant to calcium-induced terminal differentiation. The cultures were then fixed with neutral buffered formalin (an aqueous solution of 4% formaldehyde, 4 g/liter monobasic sodium phosphate, and 6.5 g/liter dibasic sodium phosphate at pH 7.4) and stained with 2 g/100 ml Rhodamine B (Sigma) in water, after which the foci were counted and sized. Our previous studies had indicated that although mouse epidermal cells grew better at 31°C than at 37°C (13), calcium selection was not as effective at 31°C because of excessive spontaneous transformation.

Injection of Cells into Nude Mice. Some of the foci from preliminary experiments with SENCAR and BALB/c (Frederick Cancer Research Facility) mice were not fixed but were subcultured, when the flasks became confluent, with a trypsin-EDTA solution consisting of 0.25 g/100 ml trypsin (Grand Island Biological Co.) and 0.2 g/100 ml EDTA (Grand Island Biological Co.) in PBS. Cells from two lines so generated from mice treated with MNNG plus four TPA treatments were later injected s.c. at 1.0 x 10^6 cells/mouse into BALB/c nude mice (Life Sciences, St. Petersburg, FL) to test for tumorigenicity, with routine histology of resultant tumors. Mice without tumors were monitored for at least 3 months.

Statistical Analyses. Appropriate transformations (square root transformation for focus counts, logarithmic transformation for focus size) were used to correct the data to meet the assumptions of analysis of variance (14). The corrected number of foci per flask was averaged over all the flasks in each group to give the number of foci per million cells plated. For sizing of foci, the corrected diameters of all foci in all flasks of a given group were averaged. The data were reported after undoing the corrections. To express the data as foci per mouse, the average number of foci per million cells after undoing was multiplied by the initial cell yield per mouse (in millions) for each group.

Mean separation subsequent to analysis of variance was done with the Student-Newman-Keuls procedure. In cases with heterogeneous variances, Snedecor’s approximate test of equality of means was used.

RESULTS

Morphology of Primary Cultures. Epidermal cells from adult mice continued to proliferate with little terminal differentiation (6) for several weeks if grown in LoCa MEM at 37°C (Fig. 1A). The cells had the distinct cobblestone appearance of epithelial monolayers without the extensive overlying keratinization char-

Fig. 1. A, adult mouse epidermal cells cultured for 6 weeks in LoCa MEM (phase contrast). The cells retained a basal cell morphology with little terminal differentiation. B, cells from carcinogen-treated mice cultured for 6 weeks in HiCa MEM (phase contrast). A few senescent cells (S) can be seen next to a focus of Ca-resistant cells with overlying keratinized debris (KD). C, fixed and Rhodamine B-stained foci from either MNNG- (M) or MNNG plus TPA-treated (MT) cells after 1 month in vitro. Flasks of untreated or TPA-treated cells rarely had any foci.

4 D. R. Miller, A. Viaje, and T. J. Slaga, unpublished observations.
characteristic of terminally differentiating epidermal cells (13). Cultures of untreated cells switched to HiCa MEM after 2 days in vitro and grown at 37°C deteriorated and eventually died. In cultures of carcinogen-treated cells, a few cells continued to proliferate after being switched to HiCa MEM, forming large foci (Fig. 1B) that could be scored after staining with Rhodamine B (Fig. 1C). Cells of these Ca-resistant foci continued to proliferate as well as differentiate, accumulating the overlying, squamous keratinization characteristic of keratinizing epidermal cells in culture (13).

Quantitation of Focus-forming Cells. The epidermis of MNNG-treated mice contained numerous Ca-resistant cells, as evidenced by focus production in vitro (Fig. 2). Although the plating density was too high to justify the use of a term other than “focus,” we assume, since the epidermal cells were dispersed before plating, that each focus is the result of a single Ca-resistant cell. The data are expressed as foci per million cells relative to the MNNG group, since the absolute number of foci detected varied from one cell preparation to another (discussed later). Cultures from either control or TPA-treated mice produced very few foci. The most interesting result here is that TPA promotion of MNNG-treated mice initially reduced the number of Ca-resistant cells below what was found with MNNG alone. As papillomas started to develop in some MNNG plus TPA-treated mice, Ca-resistant cells accumulated in the skins of those mice but not in the skins of similarly treated mice without papillomas. It is possible that this accumulation occurred in the papillomas. These differences in focus production did not appear to be due to differences in plating efficiency since differences were not apparent (data not shown) in parallel cultures maintained under nonselective conditions (in LoCa MEM).

TPA-induced reduction of Ca-resistant cells in the epidermis of MNNG-treated mice is also apparent if the data are expressed as foci per mouse (Fig. 3). This shows that TPA-induced hyperplasia was not reducing apparent focus number by merely diluting out initiated cells with more, normal cells. TPA caused an actual, early loss of these cells in the epidermis of the mouse up until papilloma formation. The data are less consistent here, because the yield of cells varied from preparation to preparation but had to be taken into account to express the data in this way. We did not therefore think a complete statistical analysis of these data would be very informative. The actual number of Ca-resistant cells per mouse is shown in Table I. The numbers again fluctuated from experiment to experiment. We have no reason to assume at this point that this was due to something other than experimental variation. Overall, the numbers of Ca-resistant cells without TPA treatment were much greater than the maximum number of papillomas (about 50) ever produced on the back of a SENCAR mouse under ideal conditions (our regimen should have yielded less than five per mouse). With TPA treatment, there were fewer Ca-resistant cells, but these cells accumulated with papilloma formation.

Growth Characteristics of Focus-forming Cells. The focus diameter (Fig. 4) gives an indication of the proliferative ability of the focus-forming cells. The results are again expressed relative to the MNNG group because of variation from one cell preparation to another. Cells from MNNG plus TPA-treated mice bearing papillomas formed foci that grew more rapidly than MNNG foci, while foci from MNNG plus TPA-treated mice without papillomas did not. TPA also caused transient, statistically significant increases in focus size after 2 and 4 weeks of treatment, indicating an early selection for more rapidly growing over more slowly growing Ca-resistant cells. Although our results do not necessarily reflect the growth rates of these cells in vivo, they do indicate certain absolute differences that exist between TPA-treated and non-TPA-treated cells.

Some of the flasks with foci from preliminary experiments were subcultured instead of fixed to allow us to examine the cells after many further divisions. One epithelial cell line that arose from BALB/c mice that had received MNNG plus 2 weeks (four treatments) of TPA was not tumorigenic at the 80th population doubling in nude mice. A corresponding line from SENCAR mice is shown in Fig. 5A. This cell line retained the differentiated epithelial morphology of cultured epidermal cells, forming flattened, cornified cells (Fig. 5B) over a stratified in vitro “epithelium” with keratin and desmosomes (Fig. 5C). This line at the 15th population doubling did not form tumors when injected s.c. into nude mice. Injected at the 102nd doubling, however, the line formed macroscopically visible cystic lesions, one of which is shown in Fig. 5, D and E. Within the
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Table 1  Cells resistant to Ca-induced terminal differentiation in the epidermis of treated mice as evidenced by focus formation in vitro

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>MNNG only</th>
<th>MNNG plus TPA</th>
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<tbody>
<tr>
<td></td>
<td>Sample size (flasks)</td>
<td>Ca-resistant cells/mouse (mean)</td>
</tr>
<tr>
<td>0</td>
<td>28</td>
<td>11</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>195</td>
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<tr>
<td>2</td>
<td>40</td>
<td>36.8</td>
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<td>4</td>
<td>40</td>
<td>140</td>
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<tr>
<td>6</td>
<td>40</td>
<td>113</td>
</tr>
<tr>
<td>10</td>
<td>18</td>
<td>67.4</td>
</tr>
<tr>
<td>10b</td>
<td>16</td>
<td>134</td>
</tr>
</tbody>
</table>

* For either untreated or TPA-treated cells, the upper limit of the 95% confidence interval was never greater than three Ca-resistant cells/mouse, the mean never greater than one. For these groups, sample size ranged from 10 to 48 and was most often around 40.

** Data derived from papilloma-bearing mice.

† Repeat of previous time point one week later.

cyst were hyperplastic masses of cells, with a well-differentiated, epithelial structure. As can be seen in Fig. 5D, the epithelial masses were surrounded by keratin that had accumulated in the cyst. Local connective tissue formed finger-like stromal projections (Fig. 5, D and E) into the epithelial tumor; however, no signs of invasion were detected in the surrounding stromal tissue. The same line injected at the 48th doubling into s.c. silicone chambers implanted onto preformed granulation tissue beds in SENCAR mice (15) formed similar noninvasive, exophytic, well-differentiated epithelial tumors.

These epithelial structures formed in both nude and SENCAR mice were essentially identical histologically to the papillomas produced in standard two-stage carcinogenesis experiments.

**DISCUSSION**

No work that has been done so far establishes exactly what Ca-resistant cells are. Whatever their nature, that they are specifically produced by carcinogen treatment alone warrants their further study. As previously mentioned, it has been suggested that they are initiated cells. As such, clonal expansion of these cells in vivo should result eventually in papilloma production, a small subset of which would go on to form squamous cell carcinomas (16). Our data indicate that papilloma-bearing skin has more of these cells than skin without papillomas, consistent with the hypothesis that these cells are initiated cells. Moreover, one cell line that we carried to a high population doubling eventually did form histological papillomas in vivo. At the same time, that line and another BALB/c line did not form tumors at a lower population doubling level. [A more extensive characterization of the in vivo growth properties of such cell lines will appear in a subsequent manuscript.] These findings indicate that the early foci that we counted in vitro, if indeed composed of initiated cells, required further changes to become tumorigenic. This phenomenon in vivo has become known as "progression."

Treatment of female SENCAR mice with 2–5 μmol MNNG followed by 2 μg TPA twice per week never produced more than three papillomas per mouse. In our experiments, we never found fewer than 11 Ca-resistant cells per mouse at any time point with MNNG treatment alone. If we assume that Ca-resistant cells are initiated cells and that there was no clonal expansion in absence of TPA treatment, then there were more initiated cells than expected based upon the papilloma data. There are two possible explanations for this: either many of the Ca-resistant cells do not give rise to papillomas (this is indeed possible), or a clonal expansion of initiated cells had already occurred prior to our sampling. The latter explanation is indeed reasonable since there was apparent toxicity at 5 μmol MNNG in a previous study, as evidenced by decreases in papilloma production and animal survival over a 2-μmol dose. In our experiments, the lowest yield of Ca-resistant cells was 1 week after MNNG treatment (Table 1, 0 weeks of TPA). Presumably, the clonal expansion due to MNNG toxicity was still taking place. This would seem to be in line with other studies in which MNNG caused an increase in DNA synthesis (after a transient 2-day drop) that did not return to normal until 11 days after treatment.

The effect of TPA on MNNG focus production has some interesting aspects. At all time points prior to papilloma development, TPA reduced the number of Ca-resistant cells due to MNNG treatment. Based upon focus size, TPA also preferentially decreased the number of the more slowly growing Ca-resistant cells...
resistant cells during the early weeks (2–4) of promotion. MNNG plus TPA-treated mice with papillomas, on the other hand, produced many more Ca-resistant cells that also grew much more rapidly. It therefore appears that there was an early TPA selection against more slowly growing Ca-resistant cells, but that either this selection was complete by week 6 or was overshadowed by the putative accumulation of Ca-resistant cells into the developing papillomas.

If we consider this argument in terms of initiation-promotion theory, then during early promotion TPA not only selects for initiated cells over normal cells, but also for more rapidly growing initiated cells over more slowly growing initiated cells. The mechanism for this selection may be through TPA toxicity (18–20), preferentially toward more normal cells (21–23); whether enhanced terminal differentiation by TPA also plays a role is unclear (24–27). Following selection, subsequent promotion would entail only general effects (e.g., an overall proliferative response to TPA toxicity) with less selective pressure. Once papillomas have formed, the cellular changes resulting in papillomatous growth are reflected in culture by an accumulation of rapidly proliferating, Ca-resistant cells.

This parallel to the recently described phenomenon of two-stage promotion (28) is intriguing, to say the least. In two-stage promotion of initiated mice, only one treatment with TPA is required for Stage I of promotion, while multiple applications (Stage II) of other, nonpromoting chemicals such as mezerein or 12-0-tetradecanoylphorbol-13-acetate (29) can complete the promotion process. Neither treatment alone is sufficient. Thus, repetitive TPA treatment effects two events (one early and one late) required for promotion. Our findings indicate that the first event (Stage I) may be a specific selection for more rapidly proliferating cells. The second event (Stage II) may be a generalized hyperplasia allowing these cells to accumulate.

ACKNOWLEDGMENTS

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REFERENCES

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