Sensitivity of Subpopulations of Mouse Skin Papillomas to Malignant Conversion by Urethane or 4-Nitroquinoline N-Oxide

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ABSTRACT

Papillomas induced in SENCAR mice by initiation with 7,12-dimethylbenz[a]anthracene and promotion by treatment for 10–12 weeks with 12-O-tetradecanoylphorbol-13-acetate (TPA) convert to malignancy at a low frequency. The rate of malignant conversion can be increased by either (a) promoting with TPA for a shorter duration or (b) treatment of papilloma-bearing mice with certain genotoxic chemicals, such as 4-nitroquinoline N-oxide (4-NQO) or urethane. The spontaneous conversion rate of papillomas promoted by 5 weeks of TPA exposure is severalfold higher than that of papillomas arising later during TPA promotion. Here, we compared the sensitivity to the converting agents 4-NQO and urethane of papillomas promoted by TPA for either 5, 10, or 20 weeks. In the mice promoted for 5 weeks with TPA, the already high spontaneous conversion frequency was increased 2.5 times by 4-NQO. A 2-fold increase was found after 10 weeks of TPA promotion. In contrast, no increase was seen with 4-NQO exposure begun after 20 weeks of TPA promotion. Similar results were found with urethane as converting agent. The sensitivity of the papillomas induced by short-term TPA treatment to induced conversion remains high even after a 16-week period without TPA treatment; when urethane exposure was delayed until week 21 after TPA promotion for weeks 1–5, a 2.4-fold increase in the conversion frequency was observed.

INTRODUCTION

Multistage skin tumor induction protocols using DMBA2 as initiator and TPA as promoter in sensitive mice produce large numbers of papillomas but few carcinomas (1, 2). The relative number of carcinomas can be increased by increasing the initiating dose of DMBA (3, 4), by using promoters which differ from TPA in their mechanism of action (5–7), or by shortening the duration of TPA promotion (7). Most, if not all, carcinomas appear to develop from papillomas (8, 9); focal areas of carcinoma in situ are seen without papillomas (7–11). Thus, by varying the protocol of papilloma induction, subpopulations of papillomas with varying potential for conversion to malignancy can be produced (7).

The malignant conversion stage of tumor progression occurs spontaneously (12) but may be enhanced by treatment of papilloma-bearing mice with genotoxic agents (13). After promotion of SENCAR mice with TPA for 10–12 weeks to produce large numbers of papillomas, repeated treatment of the papilloma-bearing mice with either 4-NQO or urethane substantially increased the rate of conversion of papillomas to carcinomas (13). The exposure of experimental animals with benign lesions to genotoxic chemicals or X-rays has been demonstrated to increase malignant conversion in the epidermis of SENCAR and CD-1 mice (14, 15) as well as in rat liver (16–18).

The purpose of this study was to determine whether all papillomas were equally sensitive to induced malignant conversion or whether specific subpopulations were more susceptible, as observed for spontaneous conversion. In mice prompted with TPA for 5, 10, or 20 weeks, weekly 4-NQO applications were begun 1 week after the last TPA treatment and continued for 20 weeks. Treatment with 4-NQO increased the frequency of malignant conversion after promotion for either 5 or 10 weeks, but not after promotion for 20 weeks. Thus, the papillomas with the highest spontaneous rate of malignant conversion are also the most sensitive to induction of malignant conversion by treatment with chemicals.

MATERIALS AND METHODS

Chemicals. 7,12-Dimethylbenz[a]anthracene was obtained from Eastman, Rochester, NY; 12-O-tetradecanoylphorbol-13-acetate was purchased from LC Services, Woburn, MA; 4-nitroquinoline N-oxide was purchased from Sigma, St. Louis, MO; urethane was purchased from MCB, Cincinnati, OH. Reagent-grade acetone, the solvent for the topically applied chemicals, was purchased from Baker, Phillipsburg, NJ.

Tumor Induction Experiments. Female SENCAR mice were obtained from the National Cancer Institute-Division of Cancer Treatment Animal Program. Groups of about 30 seven- to eight-week-old mice in the resting phase of the hair growth cycle were initiated by a single topical application of 20 μg DMBA. Promotion with TPA (2 or 2.5 μg topically once/week) was begun 1 week after initiation and continued for either 5, 10, or 20 weeks. The malignant conversion stage was accomplished by 20–32 weekly treatments with 4-NQO (250 μg topically once/week) or urethane (20 mg i.p. once/week), begun 1 week after the last TPA application. In the experiment shown in Table 3, urethane treatment of mice promoted for 5 weeks was delayed until week 21 to coincide with identical treatment of mice promoted for 20 weeks. Solutions for topical application were administered in 0.2 ml acetone. Papilloma and carcinoma counts were recorded biweekly, and mice were weighed once/month. A lesion was counted as a papilloma when it reached a diameter of more than 1 mm and was present for 2 consecutive weeks. Suspected carcinomas characterized by crusting and ulceration, with elevation of the margins of the tumor, were verified histologically by standard pathological criteria (19). Pathological evaluation of most papillomas was not performed. Since early carcinomas arising in papillomas would not have been detected, the carcinoma incidence reported may underestimate the actual incidence. The development of the first carcinoma on a mouse generally causes the animal’s death within 4–6 weeks, also resulting in a low estimate of conversion frequency (4, 7). Survival of mice varied between 87 and 100% at the time the first carcinoma developed in each group.

Individual papillomas were mapped and recorded biweekly in groups 1 and 2 of the experiment shown in Table 1. All of the 37 carcinomas in these mice arose in lesions scored grossly as papillomas, in accord with earlier reports (8, 9). Although individual tumors were not followed in mice with large numbers of papillomas, it appears likely that papillomas progress to carcinomas in these mice as well. As a measure of conversion of benign to malignant tumors, the percentage of conversion in each group of mice was calculated as the ratio of total carcinomas to total papillomas, expressed as a percentage.

RESULTS

Most papillomas induced by DMBA initiation-TPA promotion protocols are not persistent and do not convert to carci-
Thirty mice in each group were initiated with 20 μg DMBA/0.2 ml acetone at time 0 and promoted with 2 μg TPA/0.2 ml acetone weekly beginning at week 1. In groups 2, 4, and 6, 250 μg 4-NQO/0.2 ml acetone were applied weekly for 20 weeks, beginning at weeks 6, 11, and 21, respectively. The experiment was terminated at week 52.

### Table 1: Effect of duration of TPA promotion on malignant conversion by 4-NQO

<table>
<thead>
<tr>
<th>Group</th>
<th>Wk of promotion</th>
<th>Treatment (wk)</th>
<th>% with carcinomas*</th>
<th>Total carcinomas</th>
<th>Total papillomas</th>
<th>% of conversion</th>
<th>Carcinoma latent period (wk)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>Acetone (6–25)</td>
<td>52</td>
<td>25</td>
<td>189</td>
<td>13.2</td>
<td>43.8 ± 1.3</td>
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<tr>
<td>2</td>
<td>5</td>
<td>4-NQO (6–25)</td>
<td>33</td>
<td>12</td>
<td>37</td>
<td>32.4</td>
<td>32.7 ± 1.7</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>Acetone (11–30)</td>
<td>67</td>
<td>24</td>
<td>582</td>
<td>4.1</td>
<td>38.0 ± 1.7</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>4-NQO (11–30)</td>
<td>77</td>
<td>38</td>
<td>475</td>
<td>8.0</td>
<td>34.4 ± 1.0</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>Acetone (21–40)</td>
<td>67</td>
<td>24</td>
<td>748</td>
<td>3.2</td>
<td>40.7 ± 1.6</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>4-NQO (21–40)</td>
<td>82</td>
<td>30</td>
<td>869</td>
<td>3.5</td>
<td>40.1 ± 0.9</td>
</tr>
</tbody>
</table>

* Number of mice with one or more carcinomas/number of mice surviving when first carcinomas appeared × 100.² Mean time (in weeks) of appearance of each carcinoma ± SE. Group 2 differs significantly from group 1 (P < 0.0001); group 4 differs marginally from group 3 (P = 0.057); group 6 and group 5 are not significantly different (P = 0.86).

In SENCAR mice initiated with 20 μg DMBA and promoted for 40 weeks with TPA, less than 3% of the papillomas converted to malignancy within 60 weeks (20). However, shortening the duration of promotion to 10, 5, or 2 weeks increased the percentage of conversion to 3.2, 4.1, or 13.2%, respectively (Table 1). To test the populations of papillomas promoted by 5, 10, or 20 weeks of TPA for responsiveness to a converting agent, 4-NQO treatments (250 μg/0.2 ml acetone weekly) were begun at week 1 after the last TPA treatment. As shown in Table 1, exposure to 4-NQO beginning at week 11 after 10 weeks of TPA treatment reduced the papilloma incidence (14) but increased the conversion frequency to 8.0%, an increase similar to that reported previously (13, 14). In contrast, when 4-NQO was begun at week 21 after 20 weeks of TPA, the percentage of conversion was only 3.5%. When 4-NQO treatments were begun at week 6 after 5 weeks of TPA promotion, papillomas were just beginning to appear. Treatment with 4-NQO at this time reduced the number of papillomas in the group of 30 mice to only 37, but 12 of these tumors converted to carcinomas, a 32.4% conversion frequency. In the acetone-treated control mice, 189 papillomas and 25 carcinomas were seen, a conversion frequency of 13.2%. The latent period for carcinoma development was shortened by 11 weeks in the group treated with 4-NQO beginning at week 6. Carcinoma latency was unaffected by 4-NQO in mice promoted for 20 weeks and was shortened marginally in mice promoted for 10 weeks. Survival in groups of mice exposed to 4-NQO or acetone was similar either at the time when the first carcinoma appeared or at the end of the treatment period (Table 2).

Elimination of TPA promotion between the stages of DMBA initiation and conversion by 4-NQO or urethane greatly reduced the development of papillomas and carcinomas (14). In controls for this experiment, 129 mice exposed to 4-NQO for 20 weeks with no intervening TPA promotion (after DMBA initiation, 4-NQO exposure was begun at either week 1, week 6, week 11, or week 21), developed 106 papillomas, and 28 of these tumors converted to malignancy (not shown). This conversion frequency of 26.4% is comparable to that in the papillomas resulting from 5 weeks of TPA promotion. A control group of 30 mice that were not exposed to DMBA or TPA but were treated weekly for 20 weeks with 250 μg 4-NQO developed 9 papillomas and no carcinomas. At this dose and treatment schedule, 4-NQO alone is very weakly tumorigenic.

In order to verify the unresponsiveness of papillomas resulting from long-term TPA exposure, urethane was compared to 4-NQO for activity as a converting agent in mice promoted for either 10 or 20 weeks. In this experiment, the spontaneous conversion rate of 1.7% after 10 weeks of TPA was increased to 3.9% after urethane exposure and 4.6% after 4-NQO treatment (Table 3). The latent period for carcinoma development was significantly reduced by nearly 10 weeks in urethane-treated mice and by about 8 weeks in 4-NQO-treated mice. After 20 weeks of promotion with TPA, the conversion rates were 1.3% in the control group and 1.4 and 2.2% in the mice exposed to urethane and 4-NQO, respectively. The carcinoma latency was unaffected by either agent begun at week 21. Thus, a significant increase in the conversion rate and a shortening of the carcinoma latent period were found with urethane as converting agent after 10 weeks of TPA promotion. With urethane injections begun after 20 weeks of promotion, neither the conversion rate nor carcinoma latency were affected. As shown in Table 4, survival was similar for mice exposed to 4-NQO or acetone. The earlier deaths in mice given injections of urethane are the result of earlier development of carcinomas (13, 14).

To test whether the conversion-sensitive papillomas which result after 5 weeks of TPA promotion retain their sensitivity to a converting agent, two groups of SENCAR mice were initiated with 20 μg DMBA and promoted with TPA for either 5 or 20 weeks. Urethane injections were begun in both groups at week 21 and continued for 30 weeks. As shown in Table 5, with 5 weeks of promotion followed by a 16-week period with no treatment, urethane exposure began at week 21 resulted in a conversion frequency of 20.0% (1.03 carcinomas/mouse) compared to the spontaneous conversion frequency of 8.5% (0.55 carcinomas/mouse). As shown in Fig. 1, the rate of carcinoma formation was clearly increased relative to the un-treated control group within 12 weeks of the first urethane exposure. Papillomas resulting from short-term promotion with TPA do not evolve spontaneously to nonresponding papillomas; instead they retain their responsiveness to a converting agent for at least 16 weeks. As in the experiments shown in Tables 1 and 2, treatment with the converting agent did not affect the conversion of papillomas on mice promoted for 20 weeks.

Large differences in numbers of tumors and calculated values
PAPILLOMA SENSITIVITY TO CONVERSION BY MUTAGENS

Mice were initiated by topical application of 20 μg DMBA/0.2 ml acetone at time 0 and promoted with weekly applications of 2.5 μg TPA/0.2 ml for 10 or 20 weeks. Conversion was accomplished by 30 weekly exposures to urethane (20 mg i.p.) or 4-NQO (250 μg/0.2 ml acetone) beginning at week 11 or 21. The experiment was terminated at week 52.

<table>
<thead>
<tr>
<th>Group</th>
<th>Wk of promotion</th>
<th>Treatment (wk)</th>
<th>% with carcinomas</th>
<th>Total carcinomas</th>
<th>Total papillomas</th>
<th>% of conversion</th>
<th>Carcinoma latent period (wk)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>Acetone (11–40)</td>
<td>35</td>
<td>17</td>
<td>975</td>
<td>1.7</td>
<td>39.4 ± 2.6</td>
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<tr>
<td>2</td>
<td>10</td>
<td>Urethane (11–40)</td>
<td>67</td>
<td>26</td>
<td>600</td>
<td>3.9</td>
<td>29.6 ± 1.7</td>
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<tr>
<td>3</td>
<td>10</td>
<td>4-NQO (11–40)</td>
<td>67</td>
<td>27</td>
<td>594</td>
<td>4.6</td>
<td>31.6 ± 1.4</td>
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<tr>
<td>4</td>
<td>20</td>
<td>Acetone (21–50)</td>
<td>35</td>
<td>12</td>
<td>943</td>
<td>1.3</td>
<td>35.8 ± 4.3</td>
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<tr>
<td>5</td>
<td>20</td>
<td>Urethane (21–50)</td>
<td>43</td>
<td>14</td>
<td>974</td>
<td>1.4</td>
<td>36.3 ± 3.0</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>4-NQO (21–50)</td>
<td>58</td>
<td>16</td>
<td>736</td>
<td>2.2</td>
<td>38.6 ± 1.9</td>
</tr>
</tbody>
</table>

* Mean time (in weeks) of appearance of each carcinoma ± SE. The differences between group 2 and group 1 (P = 0.0031) and group 3 and group 1 (P = 0.0065) are very significant. No significant difference was seen between group 5 and group 4 (P = 1.0) or group 6 and group 4 (P = 0.55).

DISCUSSION

When papillomas with a high spontaneous conversion frequency were induced in DBMA-initiated mice by promotion with TPA for 5 or 10 weeks, subsequent treatment with either urethane or 4-NQO as converting agent increased the conversion frequency 2–3-fold and shortened the carcinoma latent period (Tables 1 and 3). In papillomas produced by 20 weeks of TPA promotion, the response to urethane- or 4-NQO-induced conversion was insignificant.

A likely explanation for these results is that there are at least 2 populations of papillomas which differ in their potential for both spontaneous and induced malignant conversion. The conversion-sensitive papillomas produced by 5 weeks of TPA promotion retain their sensitivity to conversion by urethane for at least 16 weeks in the absence of further TPA exposure (Table 5). If the potential for conversion is a stable characteristic, then continued TPA treatment for longer than 10 weeks may induce a sufficient number of nonresponding papillomas, so that those which respond to converting agents are a minority of the total papilloma population. Alternatively, long-term TPA exposure could alter the properties of the responsive population of papillomas to render them unable to convert to malignancy, perhaps by altering their ability to metabolize 4-NQO or urethane to their ultimate active forms (21, 22). In contrast to 4-NQO and urethane which are ineffective after 20 weeks of TPA, a direct-acting alkylating agent, ethylnitrosourea (23), and a physical converting agent, ionizing radiation (15), are both effective when given at this time.

The basis for the differences in potential for progression in subpopulations of papillomas is not clear. Since both the clone size of initiated cells (24) and the extent of chromosomal abnormalities within the cells (25) increase with the duration of TPA promotion, one might expect a parallel increase in the sensitivity to malignant conversion induced by genotoxic converting agents. Our results do not support this expectation; the sensitivity to converting agents seen after 5–10 weeks of promotion was not found after TPA promotion for 20 weeks. Papilloma volume, and perhaps the number of hypothetical target cells for converting agents, differed greatly among mice exposed to TPA for 5, 10, or 20 weeks. Papillomas were not yet visible after 5 weeks of promotion and were larger at 20 weeks than at 10 weeks. Thus, the dose of converting agent per papilloma cell is likely to be less in a large papilloma than in a small papilloma. Increasing keratinization seen in large papillomas also produces a physical barrier to the delivery of the converting agent to the critical tumor cells. This problem may be less important for urethane, administered i.p., than for the topical applied 4-NQO.

The production of papillomas with high spontaneous conversion potential by short-term TPA exposure after initiation with 20 μg DMBA, the dose used here, was not observed when the initiating dose of DMBA was reduced to 2 μg (4). Thus, the induction of the responding papillomas may be dependent on a sufficiently high dose of DMBA. This finding adds support to the concept that subpopulations of initiated cells exist which differ in their potential for progression to malignancy (7, 12, 26–28). The initiated cells with a high potential for progression to malignancy are characterized by their persistence and the ease with which they are promoted, either by short-term TPA (7) or by other promoters such as mezerein (7, 28) or chrysarobin (6). The unique properties of these cells and the papillomas which develop from them suggest that they may have a characteristic DNA lesion (or combination of lesions) which differs from that found in the initiated cells that produce non-progressing papillomas. The critical genes altered in the initiated cells most responsive to promotion and the converting agents 4-NQO and urethane have not been defined.

Introduction of an activated v-ras gene by direct application of the Harvey murine sarcoma virus to scarified back skin is an initiating stimulus in mouse epidermis (29). The papillomas and carcinomas which develop after TPA promotion express v-
ras mRNA and the v-ras p21 protein at elevated levels compared to the expression of c-ras in normal epidermis. Furthermore, 90% of the papillomas induced by DMBA initiation-TPA promotion protocols display a ras<sup>A</sup> gene activated by an A —¿» T transversion at codon 61 (30). Potential differences could exist in ras activation or expression in populations of DMBA-initiated papillomas promoted for short or long duration with TPA. Transfection of NIH-3T3 cells with DNA extracted from individual papillomas indicated the presence of a dominant transforming gene in 14 of 16 papillomas resulting from 10 weeks of TPA promotion (31). A similar result was found in papillomas produced by 5 weeks of promotion with TPA. Southern analysis of tumor DNA digested with XbaI indicated that the ras<sup>A</sup> gene was activated by an A —¿» T transversion at codon 61. Thus, the critical difference between populations of papillomas produced by short-term and long-term promotion does not appear to be due to a difference in ras<sup>A</sup> activation.

Keratinocyte cell lines derived from DMBA-treated epidermis or from DMBA-initiated, TPA-promoted papillomas contain a codon 61 A —¿» T transversion in ras<sup>A</sup> and produce papillomas when grafted to the backs of athymic mice (32). Transfection of the v-fos gene into cells of these ras-containing lines resulted in the development of malignant tumors on grafting (33). Under these conditions, introduction of an activated fos gene can complement an activated ras gene to accomplish the malignant conversion stage of carcinogenesis. Activation of fos in carcinomas, or in the population of papillomas with a high tendency for conversion, has not yet been studied. Similarly, introduction of the neu and p53 oncogenes into papilloma-derived keratinocytes accomplishes the first stage in producing tumors similar to in situ carcinomas (34). Other markers of malignancy, including increased levels of the proteases trans in (35) and urokinase and the loss of high molecular weight keratins (36), could also be altered in the papillomas with a high chance of progressing to malignancy. The possibility that this subpopulation of papillomas displays one or more of the markers associated with malignancy is currently being pursued in our laboratory.

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REFERENCES


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