Ionizing Radiation as an Initiator: Effects of Proliferation and Promotion Time on Tumor Incidence in Mice

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ABSTRACT

Previously we have shown that a single subcarcinogenic dose of ionizing radiation followed by 60 wk of 12-O-tetradecanoylphorbol-13-acetate (TPA) leads to the formation of squamous cell carcinoma in Sencar mice. Our previous results also indicate that TPA pretreatment prior to irradiation results in an overall increase in total tumor incidence, including both epidermal and nonepidermal tumors (D. R. Jaffe and G. T. Bowden, Radiat. Res., 106: 156–165, 1986). These studies have been expanded in CD-1 mice to further investigate the effect of the proliferative state of the skin prior to irradiation and the promotion duration after irradiation on tumor incidence. To examine the influence of the proliferative state of the skin, 17 nmol of TPA were applied to one-half of the mice 24 h prior to irradiation. The skin was irradiated using 4 MeV X-rays at a dose rate of 0.31 Gy/min. Animals received a single dose of X-rays at 0.5 or 11.25 Gy followed by twice weekly applications of TPA (8 nmol). The animals were then promoted for either 10 or 60 wk. All animals that were promoted with TPA for the same duration had a similar incidence of papillomas regardless of radiation or TPA pretreatment. Increasing the promotion duration did not significantly alter the incidence of squamous cell carcinomas at either initiation dose. At the lower initiation dose only animals that were promoted for 60 wk developed squamous cell carcinomas. TPA pretreatment at the higher dose resulted in a slight decrease in tumor incidence; however, this was not statistically significant. The incidence of basal cell carcinomas was dose dependent and appeared to be independent of TPA promotion. These data support our earlier findings that radiation can act as a weak initiator of squamous cell carcinomas and induce basal cell carcinomas in mouse skin.

INTRODUCTION

Animal studies have provided evidence which clearly indicates that ionizing radiation initiates events which are retained in viable cells for long periods of time without any evidence that the lesion(s) undergoes further change or expression until a subsequent event is induced (1–6). Early work carried out by Berenblum and Shubik (1) established that ionizing radiation was an effective initiator. Shubik et al. (3) found that exposure of mouse skin to β-rays, which alone produced no tumors during the course of the experiment, did so if the animals were promoted with croton oil.

Tumors can be induced on the backs of mice following initiation with a subcutaneously exposure to a carcinogen and subsequent repetitive applications of a promoter. Using this model system we have shown that ionizing radiation can act as an initiator in Sencar mouse skin and that TPA2 promotion enhances this effect (7). These studies have been expanded in CD-1 mice to further investigate the effect on tumor incidence of the proliferative state of the skin prior to irradiation and promotion duration after irradiation.

TPA pretreatment causes a loss of basal cells and an increase in the number of suprabasal cells (8–10). The loss of basal cells is followed by a peak of DNA synthesis at approximately 30 h and a peak of mitosis at 2 days (8). A number of laboratories have shown that the proliferative state of the target cell population can influence carcinogen-induced tumorigenesis. Increased rates of cell proliferation induced by hyperplastic agents prior to the application of a chemical initiator often result in a faster and greater response to the initiator (11–15). In fact, Hennings et al. (13, 14) have shown that the effect of TPA pretreatment on tumor incidence is dependent on the nature of the initiator. It has long been known that the sensitivity of cells to ionizing radiation varies with age in the cell cycle and that the sensitivity of a tissue is directly proportional to the rate of cellular proliferation. Thus, we were interested in examining the effect of TPA pretreatment on the tumor incidence with ionizing radiation as an initiator.

The effects of the duration of the treatment with TPA on the number and incidence of skin papillomas and carcinomas as well as the malignant conversion rate have been well documented for chemical initiators (16–19). Verma and Boutwell (18) have shown that TPA promotion for 18, 24, 30, or 36 wk elicited virtually identical yields of papillomas. The incidence of carcinomas, on the other hand, was less than maximal for mice promoted for 24 wk or longer. In fact, Hennings et al. (19) have shown that papillomas induced by the first few TPA treatments are much more likely to progress to carcinomas than those which appear later. It was of interest to us to determine the effects of promotion duration on the incidence of papillomas, carcinomas, and the malignant conversion rate in mice initiated with ionizing radiation.

An examination of the proliferative state of the skin prior to irradiation and promotion duration after irradiation will give us a better understanding of mechanisms involved in initiation by ionizing radiation. In this report, we describe the induction of squamous and basal cell carcinomas in mice initiated with ionizing radiation and how the proliferative state of the skin and promotion duration affect the tumor incidence.

MATERIALS AND METHODS

Female CD-1 mice, 5 to 7 wk of age, were used for these studies. The animals were purchased from Charles River, Boston, MA. Five animals each were housed, fed, shaved, and cared for as previously described (7).

TPA (purchased from Chemicals for Cancer Research, Chanhassen, MN) solutions were prepared in acetone and were administered to shaved backs of animals in a volume of 0.2 ml. Control mice were treated with the same volume of acetone. Animals that were pretreated with TPA were painted with 17 nmol 24 h prior to irradiation. TPA promotion was started 2 wk after irradiation. To study the consequence of promotion duration on total tumor incidence, animals were treated twice per week with 8 nmol of TPA for either 10 or 60 wk. Acetone treatment was started in Wk 11 for the group of animals treated for 10 wk with TPA and continued for the remainder of the study.

The mice were X-irradiated using a 4 MeV linear accelerator at a dose rate of 0.31 Gy/min as previously described (7). Animals were irradiated with a total dose of either 0.5 or 11.25 Gy. To examine the influence of target cell proliferation on tumor incidence, one-half of the animals in the study were pretreated with TPA prior to irradiation. The remaining half were pretreated with acetone as a control. Animals
were promoted as described above. The control group of animals were treated with acetone alone.

All groups contained between 20 and 30 animals. During the entire period of the study, cages were checked twice per week for dead or moribund animals. Once per week tumors were counted and measured, and the location was noted. It was possible to follow individual tumors over the course of the study and to visualize their progression due to the low incidence of tumors per animal. Necropsies were performed at the time of termination. Tumor sections were fixed in 10% phosphate-buffered formalin. Tissues were imbedded in paraffin wax, and 2-μm sections were stained with hematoxylin and eosin for light microscopic examination. Histopathological evaluations were made by Dr. J. N. Shively, Department of Veterinary Sciences, University of Arizona.

To investigate whether tumor incidence rates differed between treatment groups we used the χ² test (20). Time to first tumor was compared between treatment groups using the log rank test (21, 22) as per the method of Gall et al. (23). Statistical analyses were performed by Dr. Dalice Sim, Director, Clinical Trials Resources Group, John P. Robarts Research Institute, London, Ontario.

RESULTS

The results obtained from experiments involving the use of ionizing radiation as an initiator followed by various TPA promotion regimens are depicted in Fig. 1. The overall tumor incidence observed with animals that were irradiated with either 0.5 or 11.25 Gy followed by 60 wk of TPA promotion (0.36 and 0.44 tumors per mouse, respectively) was not significantly different as measured by the χ² test (P > 0.05). This was also true for animals that were promoted with TPA for only 10 wk (0.09 versus 0.08 tumors per mouse). Thus, we observed no significant (χ²; P > 0.05) dose effect between groups of animals irradiated with 0.5 and 11.25 Gy, provided that the animals received the identical promotion regimen. Animals that were not irradiated had a significantly lower tumor incidence (χ²; P < 0.01) than their irradiated counterpart. Fig. 1 also shows that the length of TPA promotion significantly influenced the total tumor incidence. However, as indicated in Table 1 this was primarily due to an increase in the incidence of papillomas and was found to be independent of radiation dose.

Although there was not a significant difference in the total tumor incidence between groups of animals irradiated with 0.5 and 11.25 Gy, distinct differences in tumor type were observed as shown in Table 1. Animals irradiated with 0.5 Gy and treated with acetone alone did not develop any tumors over the entire period of the study. A 14% incidence in basal cell carcinomas was seen in animals that were irradiated with 11.25 Gy followed by acetone treatments. As mentioned briefly above, all animals that were promoted with TPA developed papillomas. The incidence of papillomas was dependent on the length of TPA promotion and independent of radiation treatment. Unlike what is usually seen with chemical initiators we observed no regression of papillomas once they developed regardless of the length of TPA promotion. In fact animals promoted for only 10 wk developed papillomas after cessation of promotion.

Squamous cell carcinomas were only observed in animals that had been irradiated and promoted. All squamous cell carcinomas were observed to arise from preexisting papillomas with an average latency time of 16 to 22 wk following the development of a papilloma. Animals irradiated with 11.25 Gy and promoted with TPA for 10 to 60 wk developed squamous carcinomas with the same frequency. However, the papilloma incidence in these two groups was significantly different. Animals that were irradiated with 0.5 Gy and promoted for 10 wk did not develop squamous cell carcinomas. This indicated that a larger total dose of TPA was required to induce the formation of squamous cell carcinomas at the lower dose of radiation. It is also worth noting that the incidence of squamous cell carcinomas was virtually identical in groups of animals irradiated with 0.5 Gy and promoted for 60 wk versus those animals irradiated with 11.25 Gy and promoted for 10 to 60 wk (Table 1).

In Fig. 2, the effect of TPA pretreatment on the initiating potential of ionizing radiation is illustrated. The overall tumor incidence in animals irradiated with 0.5 or 11.25 Gy followed by 60 wk of promotion was 0.5 and 0.44 tumors per mouse, respectively. These values were not significantly different as measured by the χ² test, (P > 0.05). There was also no significant dose effect between any group of animals receiving the same promotion protocol. In Table 2, the influence of TPA pretreatment on animals irradiated with 0.5 or 11.25 Gy and promoted for 60 wk is presented in more detail. As shown previously, all animals irradiated and promoted for 60 wk develop squamous cell carcinomas. The highest incidence of squamous cell carcinomas occurred in animals that were pretreated and irradiated with 0.5 Gy (13%). The lowest incidence of squamous cell carcinomas was found in the group of animals pretreated and irradiated with 11.25 Gy (4%). TPA pretreatment did not significantly alter squamous cell carcinomas at either dose of radiation. Animals irradiated with 0.5 Gy and promoted for 10 wk did not develop squamous cell carcinomas regardless of TPA pretreatment. TPA pretreatment did not have any effect on latency time or tumor incidence in CD-1 mice.

As shown in Table 3 the conversion rate of papillomas to squamous cell carcinomas varies from 15 to 75% depending on treatment modality. Squamous cell carcinomas were observed in all groups of animals that were irradiated and promoted with TPA for 60 wk regardless of radiation dose. However, only animals irradiated with 11.25 Gy developed squamous carcinomas when promoted for only 10 wk. Although the malignant conversion rates are very different between animals irradiated with 11.25 Gy and promoted for 10 versus 60 wk, (75 versus 15%), the actual cumulative number of carcinomas in these two groups is identical. The cumulative number of papillomas, however, varies by a factor of 5.

In Table 4 animals were grouped according to the initiating dose of ionizing radiation they received regardless of TPA.
pretreatment and promotion duration. In this table we see that there appears to be a dose-dependent increase in the incidence of basal cell carcinomas: 0.7% for animals irradiated with 0.5 Gy and 4.2% for animals irradiated with 11.25 Gy. The incidence of basal cell carcinomas was independent of promotion time (Table 1; data not shown). The incidence of squamous cell carcinomas, however, did not appear to depend on dose. Animals treated with 0.5 Gy had a squamous cell carcinoma incidence of 5.3 versus 5.9% for animals irradiated with 11.25 Gy. However, since we do not know on which portion of the dose-response curve these two doses reside, we cannot accurately say that the incidence of squamous cell carcinomas did not appear to depend on dose. A wider range of doses of ionizing radiation needs to be studied.

A representative basal cell carcinoma, along with an accompanying histological section of the lesion, is shown in Fig. 3. Basal cell carcinomas occur rarely in mice. They are characterized by nests of small basal-type cells often consisting of ovoid nuclei with diffuse chromatin and no nucleolus.

### DISCUSSION

Hulse (24) has shown that the efficiency with which ionizing radiation acts as a complete carcinogen in mouse skin is dose dependent. Epidermal tumors had a peak yield at 21 Gy. Using ionizing radiation as an initiator we have also observed that the incidence of squamous cell carcinomas was dependent on the dose of the initiator and the duration of promotion. Given a long enough period of promotion the incidence of squamous cell carcinomas was virtually identical at 0.5 and 11.25 Gy. However, the only group of animals promoted with TPA for 10 wk to develop squamous cell carcinomas were those irradiated with 11.25 Gy. In this group the squamous cell carcinoma incidence was similar to those animals that were irradiated followed by 60 wk of TPA treatment (Table 1).

We have previously shown that, in Sencar mice, TPA pretreatment led to a significant increase in tumor incidence without an apparent effect on induction time. In our present study TPA pretreatment also did not affect the latency time. Actually, TPA pretreatment only slightly influenced the incidence of squamous cell carcinomas (Table 2). The highest incidence of squamous carcinomas occurred in animals that were pretreated, irradiated with 0.5 Gy, and promoted for 60 wk (13%). The lowest incidence was seen in animals that were pretreated, irradiated with 11.25 Gy, and promoted for 60 wk (4%). However, the effect of TPA pretreatment on the incidence of squamous carcinomas was not significant. These data suggest a possible enhancement at low-dose and a toxic effect at high-dose X-rays. Burns and coworkers (25-28) have extensively studied rat skin tumors induced by carcinogenic doses of ionizing radiation. They have stimulated rat skin cell proliferation by plucking hair repeatedly or stripping the skin surface with cellophane tape repeatedly prior to irradiation and found no difference in tumor yield (27). It is possible that the changes we observed in Sencar (7) and the differences seen in CD-1 mice (Table 2; Fig. 2) pretreated with TPA prior to irradiation are not the result of TPA-induced cellular proliferation but rather some other function of TPA. It will be of interest to examine the effect of pretreatment with nonpromoting hyper-

### Table 1 Tumor incidence in CD-1 mice initiated with ionizing radiation and promoted with TPA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>X-ray (Gy)</th>
<th>Promotion (wk)</th>
<th>% of animals with tumor</th>
<th>Tumors/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>x</td>
<td></td>
<td></td>
<td>Total</td>
<td>Papilloma</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0 (0/25)</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>10</td>
<td>4 (1/23)</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>60</td>
<td>32 (7/22)</td>
<td>23</td>
</tr>
<tr>
<td>11.25</td>
<td>0</td>
<td>0</td>
<td>14 (3/22)</td>
<td>0</td>
</tr>
<tr>
<td>11.25</td>
<td>0</td>
<td>10</td>
<td>8 (2/25)</td>
<td>0</td>
</tr>
<tr>
<td>11.25</td>
<td>0</td>
<td>60</td>
<td>44 (11/25)</td>
<td>32</td>
</tr>
<tr>
<td>0.0</td>
<td>0</td>
<td>10</td>
<td>0 (0/20)</td>
<td>0</td>
</tr>
<tr>
<td>0.0</td>
<td>0</td>
<td>60</td>
<td>26 (6/23)</td>
<td>22</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, tumor-bearing animals divided by the total animal number.
Table 3  Conversion of papillomas to squamous cell carcinoma

<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>Promotion (wk)</th>
<th>% of conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>60</td>
<td>15 (3/20)</td>
</tr>
<tr>
<td>11.25</td>
<td>10</td>
<td>75 (3/4)</td>
</tr>
<tr>
<td>11.25</td>
<td>60</td>
<td>0 (0/4)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, cumulative number of carcinomas divided by cumulative number of papillomas.

Table 4  Carcinoma incidence in CD-1 mice initiated with ionizing radiation

<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>Basal</th>
<th>Squamous</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.7 (1/144)*</td>
<td>5.3 (5/94)</td>
</tr>
<tr>
<td>11.25</td>
<td>4.2 (6/148)</td>
<td>5.9 (6/101)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, tumor-bearing animals divided by the total animal number.

Fig. 3. Photograph of a mouse with a large basal cell carcinoma (A). Photomicrograph (× 40) of hematoxylin-eosin-stained section of the lesion (B). The basal cell carcinoma is characterized by nests of small basal-type cells containing ovoid nuclei with diffuse chromatin and no nucleolus.

plastic agents and further examine the observed strain differences.

Epidermal papillomas are the most frequently observed type of lesion produced in mouse skin as a result of the initiation-promotion protocol. This has been shown for many different chemical initiators (reviewed in Refs. 29 and 30) as well as for initiating doses of UV and ionizing radiation (7, 31, 32). Burns et al. (16) have shown that greater than 90% of epidermal carcinomas develop from preexisting epidermal papillomas. However, the conversion of papillomas to the malignant phenotype is only 7 to 10% using chemical initiators. It was of interest to find a malignant conversion rate between 33 and 45% in our previous study using Sencar mice and ionizing radiation as an initiator (7). In the present study we observed a malignant conversion rate of 15 to 75% depending on treatment modality (Table 3). Although the malignant conversion rate was very different between animals irradiated with 11.25 Gy and promoted for 10 or 60 wk (75 versus 15%), the actual cumulative number of carcinomas in these two groups was identical. The cumulative number of papillomas, however, was quite different (Table 3). Using chemical initiators in this model system, it has been shown that papillomas induced by the first TPA treatments are more likely to progress to carcinomas than those that appear later (19). Our results are in agreement with these published results and support the contention that conditional papillomas contribute very little to the carcinoma yield. Thus, it would appear from these results and those presented in the literature for chemical initiators that the proportion of conditional versus autonomous papillomas is independent of the type of initiator, provided that TPA is used as the promoting agent.

At a dose of 0.5 Gy there appears to be a correlation between promotion duration and the carcinoma incidence. This is not the case for animals irradiated with 11.25 Gy. At this dose the longer promotion period resulted only in a higher incidence of papillomas. It will be of interest to examine promotion times less than 10 wk. We have shown that the development of squamous carcinomas requires irradiation followed by TPA promotion. However, the amount of TPA promotion actually required for malignant tumor formation remains to be determined.

This is one of the first reports of basal cell carcinomas occurring in mice exposed to the initiation-promotion regimen (7). Basal cell carcinomas occur rarely in experimental animals. The exception is rat skin exposed to carcinogenic doses of ionizing radiation (24). In our experiments the development of basal cell carcinomas was dependent on the dose of ionizing radiation and independent of TPA promotion. This is in contrast to squamous cell carcinomas whose formation was dependent on the interaction of ionizing radiation and TPA. Basal cell carcinomas are cutaneous epidermal tumors characterized by nests or sheets of small basal-type cells having relatively large oval basophilic nuclei (Fig. 3). They are a slow growing tumor and are the most abundant form of skin cancer in humans (33, 34). Squamous cell carcinomas, on the other hand, are more rapidly growing tumors consisting of epidermal keratinocytes characterized by invasive nests of prickle cells showing variable central keratinization (33, 35). The mechanism of induction of these two malignant tumor types and how ionizing radiation interacts with the skin to induce these lesions remain unknown. A more fundamental question is to address the nature of the target cells for these two lesions. It is known that 10% of the cells in the basal layer of mouse skin are clonogenic. These stem cells will divide, and the daughter cells which are "programmed" to differentiate will divide 2 additional times before their daughters migrate into the spinous layer (36). Are cells that have been "programmed" to differentiate, but still reside in the basal layer, the target cells involved in the induction
refers to the two major categories of skin cancers, basal and squamous cell carcinomas. Are the more immature stem cells targets for basal carcinomas? Is it possible that the target cells for these two types of cancer are the same and that it is the way the cell interacts with a carcinogen that determines the differentiation state of the tumor? These are very interesting questions that need to be examined.

The results presented here confirm earlier findings that ionizing radiation is a weak initiator in mouse skin. Our data indicate that the interaction between ionizing radiation and TPA in vivo, analyzed in terms of tumor response, is complex. The final outcome is dependent on the total dose of irradiation delivered to the dorsum, length of promotion, and the target cells involved in the tumor response.

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