Sequential Studies of Skin Tumorigenesis in Phosphoglycerate Kinase Mosaic Mice: Effect of Resumption of Promotion on Regressed Papillomas

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ABSTRACT

Most mouse skin papillomas induced by 7,12-dimethylbenz(a)anthracene initiation followed by 12-O-tetradecanoylphorbol-13 acetate (TPA) promotion are benign promoter-dependent papillomas which regress after cessation of promotion, but some benign tumors (promoter-independent papillomas) do not regress, and a few carcinomas seem to develop from progressive growth of these tumors. We have tested whether a second course of TPA promotion induces regeneration in regressed promoter-dependent papillomas and advances them to malignancy. The regression and regeneration of these papillomas were determined by serial photographs, measurements of coordinates, histopathological evaluation, and X-chromosome-linked phosphoglycerate kinase enzyme cellular markers. Most of the regressed promoter-dependent papillomas did not regenerate. However, the second course of TPA promotion induced rapid development of many new papillomas, some of which advanced to carcinomas. This finding suggests that there are more abnormal cells in the initiated mouse skin than those detected with a single course of TPA promotion.

INTRODUCTION

The two-stage mechanism of induction of benign skin papillomas with chemical carcinogens has been studied extensively in mice (1-3). However, the relationship between these benign papillomas and the malignant carcinomas that eventually develop in this system has not been precisely defined. Skin papillomas induced by an initiation-promotion regimen are heterogeneous in their ability to progress to carcinomas; most of them are promoter dependent and regress after termination of promotion (4); the remaining tumors are promoter-independent papillomas and some of these progress to carcinomas. Many investigators believe that the promoter-independent papillomas develop from preexisting promoter-dependent papillomas after repeated promotion (5, 6). However, it has been suggested recently that they form directly without an intervening promoter-dependent papilloma stage as a result of alterations induced by the carcinogen at the time of initiation (7). According to the latter hypothesis, the initiating event(s) induced by the carcinogen is critical for determining whether a papilloma progresses to a carcinoma; while promotion is necessary to facilitate the growth of initiated cells to become visible papillomas, it may not be sufficient to advance promoter-dependent papillomas to promoter-independent papillomas and finally to carcinomas.

To study the role of promotion in the progression of papillomas to carcinomas, we investigated the ability of papillomas that regress after termination of promotion to regenerate and progress to malignancy after resumption of promotion. The regression and regeneration of promoter-dependent papillomas were determined by serial photographs, measurements of coordinates, histopathological evaluation, and X-chromosome-linked PGK enzyme cellular markers (8). The results show that very few regressed papillomas regenerated after a second course of promotion. However, the additional promotion induced many new papillomas and some of these were capable of progressing to promoter-independent papillomas and carcinomas. Furthermore, the new papillomas appeared more rapidly during the second promotion, as if the first course of promotion had increased the sensitivity of the mice to a second course of promotion.

MATERIALS AND METHODS

Animals. The BALB/c PGK mosaic mice and the breeding procedures have been described (9). Eight- to 12-week-old mice were shaved 72 h prior to treatment with carcinogen and only animals showing no hair growth were used.

Tumor Kinetics. All mice with tumors measuring more than 1 mm in diameter were enumerated once a week for estimation of tumor susceptibility (the number of mice with tumors divided by total number of mice alive in the group) and r50. The papilloma frequency was estimated by dividing the total number of papillomas by the number of mice alive in each group.

Experimental Protocol. Fifty BALB/c heterozygous mice were initiated by treating the skin with 200 µg of DMBA (Aldrich Chemicals, Milwaukee, WI) dissolved in 0.2 ml acetone. This initiating dose of DMBA alone is not tumorigenic in these mice (10). However, 15 weeks of repeated TPA (10 µg) promotion three times a week at the site of initiation was found to induce tumors in more than 80% of mice, with a tumor frequency of 10–15 papillomas per animal. Continuous promotion for more than 15 weeks was found to inhibit the growth of papillomas in these mice.

Two weeks after initiation the mice were randomly divided into two groups and were treated as follows (Fig. 1). Group 1 animals were promoted by applying 10 µg of TPA in 0.2 ml acetone three times a week for 15 weeks to induce skin papillomas. The location and PGK phenotype of all papillomas were determined by serial photographs, measurements of coordinates, histopathological evaluation, and X-chromosome-linked PGK enzyme cellular markers. The results show that very few regressed papillomas regenerated after a second course of promotion. However, the additional promotion induced many new papillomas and some of these were capable of progressing to promoter-independent papillomas and carcinomas. Furthermore, the new papillomas appeared more rapidly during the second promotion, as if the first course of promotion had increased the sensitivity of the mice to a second course of promotion.

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Removal of Independent Papillomas. Ten weeks after termination of the first course of TPA promotion, mice were anesthetized by i.p. injection of sodium pentobarbital (1 mg/g body weight) (Veterinary Laboratories, Inc., Lenexa, KS), and all the remaining papillomas were surgically excised together with the surrounding skin. The wounds were immediately closed with wound clips.
EFFECT OF PROMOTION ON REGRESSED PAPILLOMAS

Fig. 1. Protocol of induction of skin papillomas in BALB/c heterozygous mice. Both groups of mice were initiated with a single application of 200 μg of DMBA in 0.2 ml of acetone 2 weeks prior to starting first course of promotion. □, 0.2 ml of acetone, three times a week; ▲, 10 μg of TPA in 0.2 ml of acetone, three times a week.

Fig. 2. Effect of two courses of promotion on tumor susceptibility and t50. □, Group 1, TPA-TPA; ▲, Group 2, acetone-TPA.

Regeneration of Regressed Papillomas. This was studied by tracking individual papillomas by location, histopathology, and PGK phenotyping as described (8). Briefly, to map the location of tumors two methods were used. First, the back of each mouse was photographed with a Polaroid camera. Second, the coordinates of each papilloma were measured. Even though surgical excision of some papillomas created slight distortions of the skin on the backs of the mice, the combination of photography and measurement of coordinates of the tumors allowed us to follow their fate during regression and regeneration.

A tumor that appeared during the second course of promotion was considered “new” if it developed at a site where there was no evidence of the existence of a papilloma prior to regression. PGK phenotyping was used to determine if a tumor that appeared at or near a site previously occupied by a neoplasm was new. Thus, a tumor was also considered new if it developed at a former papilloma site but exhibited a different PGK phenotype. A tumor was considered to be possibly “recurrent” if it developed at the location of a regressed papilloma and also exhibited the same PGK phenotype as the regressed papilloma.

Statistical Tests. The x2 test with Yates’ correction was used to determine the statistical significance of differences in tumor susceptibility. Student’s t test was used to determine the significance of differences in tumor frequency.

RESULTS

Five mice in Group 1 and one mouse in Group 2 died during anesthetization for biopsies of papillomas or surgical excision of promoter-independent papillomas. These mice were excluded from determinations of tumor susceptibility, t50, and tumor frequency.

Tumor Development. During the first course of promotion, all mice in Group 1 developed papillomas and the t50 was 7 weeks (Fig. 2). The papilloma frequency in this group at the end of the first course of 15 weeks of TPA promotion was approximately 15. Single PGK types were found in 149 of the 159 papillomas more than 2 mm in size (63 showed type B PGK and 86 showed type A). Double-enzyme PGK types (both A and B enzymes) were observed in the 10 remaining tumors. Treatment with acetone during the first course of “promotion” in controls (Group 2) did not induce tumors.

There were no statistically significant differences in the distributions of PGK types of papillomas that appeared during the second course of promotion in Group 1 versus Group 2 mice (or in either of these distributions versus that found during the first course of promotion in Group 1 animals) (Table 1). However, during the early stages of the second course of promotion, there was a significant difference in susceptibility to papilloma formation between the two groups (P < 0.01) at 5 weeks into the second course of promotion (Fig. 2). In Group 1, the first 15 weeks of TPA promotion appeared to hasten the appearance of papillomas during the second course of TPA promotion. Although none of the mice exhibited papillomas by the end of 3 weeks of promotion, the t50 was 4 weeks and 100% of the mice developed papillomas by the end of 5 weeks. In contrast, Group 2 control mice, which received acetone instead of TPA during the first course of promotion, showed a t50 of about 6 weeks. These mice exhibited during the second course of TPA promotion only 12 and 30% susceptibility by the end of 4 and 5 weeks, respectively; not until 8 weeks did 100% of the animals develop papillomas. Thus, the data suggest that the first course of TPA promotion increased the susceptibility of mice to the second course of promotion.

There were also significant differences in the frequencies of papillomas between the two groups early in the second course of TPA promotion. For example, by the end of 4 weeks of the second TPA promotion, the frequency of papillomas in Group 1 was 7.0 as compared to 0.5 in Group 2 (P < 0.01) (Fig. 3). The papilloma frequency in Group 1 peaked early, reaching a
EFFECT OF PROMOTION ON REGRESSED PAPILLOMAS

maximum of 10 by the end of 7 weeks of TPA promotion. Thereafter it remained constant for another 4 weeks and then started to regress despite another 4 weeks of TPA promotion. As in Group 1, papillomas in Group 2 also regressed but only after termination of TPA promotion. The finding that Group 1 animals developed papillomas at a faster rate during the second course of TPA promotion than did Group 2 mice also suggests that 15 weeks of prior TPA promotion increased the sensitivity to papilloma formation of the mice in Group 1.

Regeneration and Progression of Regressed Papillomas. The location, histopathology, and PGK phenotype of each papilloma more than 2 mm in diameter induced by the first TPA promotion were recorded. This information allowed us to determine which papillomas regressed spontaneously after termination of the first course of TPA promotion, and which papillomas regrew after promotion and were considered to be new growths. The other 10 tumors exhibited PGK phenotypes similar to those of the original neoplasms; some of these neoplasms may have been true recurrences. Five of these 10 "recurrent" tumors progressed to promoter-independent papillomas, 2 converted to squamous cell carcinomas, and 3 regressed by the time of autopsy. These results indicate that the great majority of spontaneously regressed papillomas do not regenerate to visible papillomas during or after a second course of TPA promotion.

Incidence and Progression of New Papillomas. Although the great majority of papillomas that appeared during the first course of TPA promotion in Group 1 had regressed spontaneously and did not regenerate during the second promotion, many new papillomas appeared during the second course of TPA promotion. The 60 new papillomas more than 2 mm in diameter were followed until autopsy (Fig. 4). Of these, 24 (40%) progressed to become promoter-independent papillomas and 1 (2%) converted to a carcinoma; the other 35 regressed by the time of autopsy. In Group 2 control mice, of the 135 papillomas more than 2 mm in diameter that appeared during the second course of TPA promotion, 32 (24%) became promoter-independent papillomas and 6 (4%) became carcinomas. The remaining 97 papillomas regressed by the time of autopsy. These results demonstrate that the second course of TPA promotion in Group 1 animals induced many new papillomas and some of them advanced to promoter-independent papillomas and one converted to a carcinoma. However, the frequency of independent papillomas and carcinomas in Group 1 during the second course of TPA promotion was not significantly different than the frequency in Group 2, which received the first course of TPA promotion 32 weeks later than Group 1.

Occurrence of Papillomas at Sites of Surgical Excision. Three promoter-independent papillomas regrew at sites where promoter-independent papillomas had been excised and each one had a PGK phenotype similar to that found in the original papilloma. Thus, the regrowth of these promoter-independent papillomas may have occurred because the surgical excision of the tumors was not complete. The relative absence of new papillomas around the sites of excision suggests that wound healing did not create a promoting stimulus that caused the growth of new papillomas.

Histology of Papillomas. No differences were noted in the histological appearances of the papillomas that occurred during the first course of TPA promotion as compared with those that occurred during the second course of TPA promotion. All papillomas were found to contain multilayered hyperplastic squamous epithelium surrounding a sparse stroma containing mesenchymal cells, lymphocytes, and macrophages.

DISCUSSION

One finding of the present studies is that in Group 1 animals the first 15 weeks of TPA promotion apparently increased the sensitivity of the initiated mice to a second TPA promotion. They developed tumors earlier and more rapidly than did the controls. For example, by the end of 4 weeks of promotion, 50% of experimental mice (Group 1) developed papillomas in contrast to 13% of control mice (Group 2) \( (P < 0.05) \) (Fig. 2). This difference in tumor susceptibility is even more notable at the end of 5 weeks of TPA promotion \( (P < 0.001) \).

It is possible that the surgical manipulation itself was responsible for the enhanced occurrence of new papillomas during the second course of TPA promotion in Group 1. For example, wounding 1 week prior to promotion has been shown to enhance the activity of a weak promoter such as 12-O-retinylphorbolester-13-acetate (11). It is less likely that wounding enhanced the development of new papillomas in the present studies, since the second promotion was not resumed until 5 weeks after wounding. Another possibility is that the trauma and stress of surgical excision of papillomas influenced the growth of papillomas after promotion was resumed. Further studies are necessary, such as those using sham skin-excised mice, to elucidate the mechanism by which the first course of TPA promotion influences the rapid formation of new papillomas after a second course of promotion with TPA.

Following individual papillomas with the parameters of location, histopathology, and PGK phenotype allowed us to determine whether the tumors that appeared during the second course of promotion were regrowths of promoter-dependent papillomas that regressed after termination of a first course of TPA. The results show that very few of the regressed papillomas regrew during or after termination of the additional promotion with TPA. It is important to note that if the sole criterion of regeneration of quiescent papillomas during the second course of promotion had been the number of papillomas that appeared, different conclusions would have been reached.

This is because the additional promotion induced many new papillomas. Thus, these findings are not consistent with the traditional concept that progenitor cells of regressed papillomas remain quiescent in the skin when promotion ceases and regenerate with additional promotion and advance to carcinomas (2, 12).

The reason(s) for the loss of the ability of regressed promoter-dependent papillomas to regenerate with a second course of TPA promotion and the factors that determine whether a papilloma will be promoter dependent or promoter independent are not known. One possibility is that these alternatives are determined solely at initiation. For example, promoter-dependent papillomas may develop from cells that are at putative "low levels of initiation" (7), and TPA may be incapable of advancing these to carcinomas (13). Alternatively, TPA promotion itself may be the critical factor in determining progression of papillomas to carcinomas. Some of the many new papillomas induced by the second course of TPA promotion advanced to promoter-independent papillomas and to carcinomas. The hy-
EFFECT OF PROMOTION ON REGRESSED PAPILLOMAS

Fig. 4. Sequential photographs of a BALB/c PGK heterozygous mouse with skin papillomas and carcinomas. The number and PGK phenotype of each papilloma on the back of the mouse are shown with leaders. a, at first biopsy performed at the end of 15 weeks of TPA promotion. b, at excision of promoter-independent papillomas performed 10 weeks after termination of the first course of TPA promotion. c, at second biopsy performed at the end of the second course of TPA promotion. d, at autopsy performed 32 weeks after second biopsy. Note the regression of papillomas 1, 2, 5-8, and 10 by the end of 10 weeks after termination of the first course of promotion. These papillomas did not regenerate during or after the second course of TPA promotion. Papillomas 3, 4, and 9 changed PGK phenotype. Papilloma 12 occurred after termination of the first course of promotion. All these papillomas were surgically removed. Papillomas 13 and 14 developed during the second course of TPA promotion. Papilloma 14 remained benign and promoter independent, and papilloma 13 progressed to a carcinoma by the time of autopsy.

The data can be explained by assuming that the carcinogen-induced initiating events are changes in the genes in the initiated cells that control their proliferation, differentiation, or both (14–16). In this way the initiated cells might become unresponsive to the growth regulatory factors of the host and thus remain quiescent. This interpretation is consistent with the irreversible and long-lasting nature of initiating events demonstrated previously (17–19) and in the present investigations. TPA, which has been shown to elicit a wide range of biological and biochemical responses (20), may overcome the quiescent state in some of the initiated cells, permitting them to proliferate. With minimal promotion, cells at an advanced level of initiation may progress to promoter-independent papillomas and carcinomas. However, the same promotion regimen may be incapable of advancing cells at lower levels of initiation beyond the benign promoter-dependent papilloma stage. The progressive growth of these latter papillomas may thus require higher doses and continued exposure to TPA promotion. It is also possible that some promoter-dependent papillomas advance to promoter-independent papillomas and finally to carcinomas as a result of specific direct tumorigenic effects of TPA or of stochastically occurring carcinogenic events of spontaneous origin. If TPA promotion is terminated prior to occurrence of such rare events in promoter-dependent papillomas, reproductive death of the abnormal cells may occur and result in regression and complete elimination of the tumors. The present data showing a rapid regression of many papillomas after cessation of promotion and their inability to regenerate during a second TPA promotion are consistent with this hypothesis.

On the other hand, under some circumstances TPA may inhibit tumor growth. Inhibition of growth of papillomas with prolonged periods of TPA promotion has been observed in the CD-1 strain of mice (21), as well as in the BALB/c PGK heterozygous animals we studied (data not shown). Thus, it is possible that prolonged exposure to TPA is toxic to some papilloma cells, resulting in regression of the tumors rather than progression to malignancy.

As discussed above, most of the papillomas that appeared in Group 1 mice during the second course of promotion were new tumors and not regenerated previously noted tumors. It is possible that these new papillomas existed as microscopic tumors in the skin during the first course of TPA promotion, remained dormant after termination of promotion, and then progressed rapidly to become visible papillomas with resumption of TPA promotion. The existence of such microscopic tumors in initiated mouse skin has been shown previously, but only during active TPA promotion (4); all of these papillomas disappeared within 3 weeks after termination of promotion. Furthermore, our observations of the regression of all detected small papillomas (less than 1 mm size) and of the great majority
of tracked papillomas (more than 2 mm size) after termination of TPA promotion, and the inability of most of these regressed papillomas to grow back during the second course of TPA promotion, argue against the possibility that new papillomas were present as microtumors after termination of the first course of promotion. The results of our experiments suggest that there are at least two different classes of initiated cells. One class evolves into papillomas during the first course of TPA promotion and the other evolves during the second course of TPA promotion. In a previous study, we reported the existence of another class of initiated cells that grow as delayed promoter-independent papillomas after termination of the first course of TPA promotion (8). Each of these papilloma types appears to have a different potential to advance to carcinomas. Other investigators also have shown differences in the malignant potential of initiated cells that grow as papillomas (5, 22, 23). As discussed previously (23), the differences in the initiated cell populations may be due to the existence of heterogeneity in the target cells, differential effects of initiating agent, or both. However, regardless of the explanation, the results indicate that there are more initiated cells in the skin of the mice we studied than can be estimated on the basis of the number of papillomas appearing during one course of promotion.

In the present studies, the detection of this large pool of initiated cells may be the consequence of using an initiating dose of DMBA that is 10-fold higher than generally used in some other strains of mice (24, 25). We used the higher dose because BALB/c mice are relatively resistant to the action of DMBA in the two-stage system of skin tumorigenesis (26). In any event, since many initiated cells did not grow as papillomas during the first course of TPA promotion, the conclusion that there are more initiated cells than would have been detected after only one course of promotion remains valid, at least for the mice we studied. It is possible that in other strains or in BALB/c mice given lower doses of DMBA most, if not all, initiated cells that can form papillomas will do so with a single course of TPA promotion. We are currently testing the latter possibility.

In summary, the present studies provide new information about the mechanism of mouse skin carcinogenesis induced by initiation-promotion protocols, at least in the experimental conditions used in these studies. First, the great majority of regressed promoter-dependent papillomas do not regenerate after additional promotion. This fact would not have been appreciated if the only criterion of regeneration had been enumeration of papillomas that appeared during additional promotion. Many such papillomas appeared, but they were new and not derived from previously regressed tumors. Second, the first promotion enhances the rapid development of new papillomas induced by the second promotion. Third, there are many more abnormal cells in the initiated mouse skin than those detected with a single course of promotion and these cells are capable of forming papillomas. Further experiments are necessary to define the mechanism of regression and progression of mouse skin papillomas.

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