The Nature of the Epidermal Growth Produced by the First Application of 12-0-Tetradecanoyl-Phorbol-13-Acetate on the Skin of Mice Initiated with Dimethylbenzanthracene

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One of the important areas of investigation today concerning skin chemical carcinogenesis in mice is the nature of the epidermal growth produced by the application of a tumor promoter on initiated mouse skin. We have investigated the kinetics of epidermal growth following a single application of 17 nmoles of 12-0-tetradecanoyl-phorbol-13-acetate (TPA) in the back skin of CD-1 female mice initiated with 200 nmoles of dimethylbenzanthracene (DMBA).

Within 5 hr after the application of TPA there is an increase in epidermal wet weight and total protein (protein/unit area of epidermis) which reached their peak of 3 to 4× that of normal between 3 and 4 days. Total DNA begins to significantly increase somewhat slower than epidermal wet weight and total protein, but reaches its peak of about 2× increase above normal by day 2. All these parameters remain elevated until 7 days after TPA treatment when they begin to return toward normal levels. But, by day 10, the end of the experimental period, they are still significantly elevated above normal levels. Within 3 hr after TPA application, a slight hyperplasia is seen, as evidenced by small increases in the number of nucleated cell layers, the total number of epidermal nuclei/mm interfollicular epidermis (IFE) and the number of suprabasal nuclei/mm IFE. This modest hyperplasia is transient and is lost by 8 hr. Then the principal hyperplasia appears with marked increases in the number of nucleated cell layers, the total number of nuclei/mm IFE and the number of suprabasal nuclei/mm IFE which by day 2 reach their peak of about 2-3× normal. During the transient hyperplasia and the early phase of the principal hyperplasia one sees considerable epidermal cell damage as evidenced by cytoplasmic vacuolization, nuclear pyknosis, and the separation of the epidermis from the dermis. This period of damage is associated with a decrease in the number of basal nuclei/mm IFE. A small increase in mitotic activity of the basal epidermal cells is seen at 3 hr at the time the transient hyperplasia occurs. Mitotic activity decreases to below normal levels by 5 hr, and then, as the principle hyperplasia begins to be produced, epidermal mitotic activity increases markedly, reaching a peak at day 1.

A comparison of the epidermal growth produced by the first application of TPA in initiated mouse skin with that in normal mouse skin, suggests that the overall growth produced by the first application of TPA in initiated mouse skin is of greater duration than that produced by TPA in normal mouse skin.

Chemically induced epidermal carcinogenesis is usually divided into 2 stages, initiation and promotion (For recent reviews, see references 1,2). Initiation presumably involves the conversion of at least some epidermal cells into latent neoplastic cells. Promotion allows for the expression of this neoplastic change. The mechanism of tumor promotion is an important area of investigation in skin carcinogenesis today. Promotion is a complicated process, which in all likelihood can be divided into a number of stages[3]. One prerequisite for our understanding of the mechanism of promotion in chemically-induced skin carcinogenesis is an understanding of the nature of the epidermal growth following the application of the chemical promoters, and indeed this has received considerable attention [1,2]. Surprisingly, there is no systematic analysis of the kinetics of epidermal growth following the application of a promoter on initiated skin of mice. For example, we know nothing about the changes in epidermal mass and in the number of basal and suprabasal epidermal cells during the production and regression of the epidermal hyperplasia induced by promoters in initiated skin of mice. In this paper, we report the results of our investigation of the effects of a single application of 17 nmoles of 12-0-tetradecanoyl-phorbol-13-acetate (TPA) on the kinetics of epidermal hyperplastic growth and regression in the skin of CD-1 female mice initiated 7 days earlier with 200 nmoles of dimethylbenzanthracene (DMBA). We also relate the kinetics of epidermal growth to the changes in epidermal mitotic activity, and in turn, these changes are related to the histological changes observed during epidermal hyperplastic growth.

MATERIALS AND METHODS

CD-1 female mice, approximately 40 days of age, were purchased from Charles River Farms (Wilmington, Mass.). The mice were kept in an air-conditioned animal room with a 12-hr light/dark cycle. Food pellets and water were available ad libitum. The mice were allowed approximately a 2-week period for acclimatization before being placed in an experiment. At this time, the skins on the backs of the mice were usually in the resting phase of the hair growth cycle [4]. Mice were clipped, and 2 days later 290 nmoles of dimethylbenzanthracene (DMBA) (Eastman Kodak, Rochester, New York) was applied in 0.2 ml of acetone (ACS reagent grade, Fisher, Rochester, New York). One week after DMBA treatment 17 nmoles of 12-0-tetradecanoyl-phorbol-13-acetate (TPA), (purchased from Dr. Peter Borchert, Chemical Carcinogenesis, Eden Prairie, Minnesota 55344), was applied to the backs of the mice. Application of DMBA or TPA was always done between 8 and 9 AM. Mice used for mitotic and nuclear counts were injected with colchicine at 9 AM and sacrificed 5 hr later as previously described [4]. Six mice were sacrificed for each time point. The techniques for epidermal nuclear and mitotic counts have been described [4].

The techniques for separating the epidermis from the dermis and measuring epidermal wet weight, protein, and DNA have been described [4,5]. Briefly, the back skin of 9 mice was clipped and a depilatory (Surgex, Cooper Scientific Corporation, Wayne, N.J.) was applied for 2 min, and then removed by thoroughly washing under running tap water. The back skin was cut out and placed on a Petri dish filled with ice, epidermis side down. The subdermal tissues of the skin were removed by scraping with a #10 blade [4]. The epidermis was scraped from the underlying dermis using a fresh #10 blade, and then dried in between 2 layers of filter paper until it reached a constant weight [4]. Epidermal homogenates, 4% w/v were prepared from the pooled epidermis of 9 mice in 0.25 M buffered sucrose as already described [4,5]. The DNA was extracted using the Schmidt-Thanhauser...
ERMINATIONS are in parentheses.

Error of the mean of the normal value

12-nmol dmab-17nm TPA 7 days after initiation is 0.167 even by the end of the experimental period at day 10, it is over 2× greater than normal, which is highly significant (p < 0.001). Unlike wet weight and total protein, the increase in total DNA, that is DNA/gm epidermis (Fig 1), is not significantly increased until 19 hr (p < 0.01). Also, the maximal increase in DNA is not as much as that of either wet weight or total protein, suggesting that some of the increase in epidermal wet weight and total protein may be due to an increase in epidermal cell size and/or hyperkeratosis. Both are observed in histological sections (see below).

Figure 2 indicates that as the epidermis increases in mass, there is an increase in the number of nucleated cell layers. This increase roughly parallels the percent increase in total DNA. Also roughly paralleling the percent increase in total DNA is the percent increase in the total number of epidermal nuclei/mm IFE (IFE), in CD-1 female mouse skin after a single application of 17 nmoles 12-0-tetradecanoyl-phorbol-13-acetate, 7 days after initiation with 200 nmoles dimethylbenzanthracene. The vertical bars represent the standard error of the mean. Six mice were used at each interval.

Figure 3 demonstrates that there is a small, but significant increase in mitotic activity of the basal cells at 3 hr which then decreases. By 18 hr, the second and major significant increase (p < 0.001) in the mitotic activity in the basal cells of the epidermis occurs. It reaches a peak at day 1, then fluctuates somewhat, but it is still significantly increased (p = 0.01) above normal by day 3. At 5 days there is a further decrease in mitotic activity, but it is still significantly above normal (p < 0.01).

Cell mitotic activity is back to normal levels by day 10. An increase in the mitotic activity of the suprabasal cells is also seen although it is much smaller than that of the basal cells, reaching a peak by day 1 (p < 0.01) and then slowly decreases to normal levels by 10 days, the end of the experimental period.
Figure 3. The mitotic activity of epidermis of CD-1 female mice after a single application of 17 nmoles 12-0-tetradecanoyl-phorbol-13-acetate, 7 days after initiation with 200 nmoles dimethylbenzanthracene. Vertical bars represent the standard error of the mean. Six mice were used at each interval. Mice were injected with Colchicine 5 hr prior to sacrificing.

Figure 4 shows a section of mouse skin one day after initiation with DMBA. It appears essentially normal. Within 8 hr after TPA treatment of DMBA initiated skin, the epidermis is thicker and there is some cytoplasmic vacuolization (Fig 5). By 18 hr there are areas of epidermis which show considerable damage (Fig 6) although the epidermis is hyperplastic. Areas can also be found which show much less damage, are hyperplastic, and show hyperkeratosis. By day 1 the epidermis is quite thick, there are abundant mitotic figures and the cells are enlarged and basophilic (Fig 7).

**DISCUSSION**

This paper presents for the first time the kinetics of epidermal growth in the skin of mice following a single application of 17 nmoles of TPA on the skin of mice initiated 7 days earlier with a single application of 200 nmoles of DMBA. It is clear that there is a rapid and pronounced epidermal growth which is detectable soon after the application of TPA.

It is instructive to compare the kinetics of TPA-induced epidermal growth in the skin of mice initiated with DMBA, with TPA-induced epidermal growth in the skin of normal mice [10]. During the first 2-3 days following the application of 17 nmoles TPA on either normal [10] or initiated mouse skin, the kinetics of overall epidermal growth are essentially the same. This is true, irrespective of whether one considers the changes in epidermal wet weight, total protein or DNA. However, after 3 days there is a significant decrease in epidermal mass in normal skin treated with TPA [10]. In contrast, the epidermis of DMBA initiated skin does not significantly decrease in mass at 3 days. Indeed, epidermal mass remains elevated until 7 days, and then it begins to return toward normal levels. Similarly the total number of epidermal nuclei/mm IFE also remain significantly elevated from 3 to 7 days following treatment with TPA in initiated mouse skin, in contrast to normal mouse skin in which at 3 days the number of epidermal nuclei/mm IFE is...
reduced [10]. Thus, a single application of 17 nmols of TPA in initiated mouse skin results in a prolonged maintenance of the epidermal hyperplasia compared to that produced by TPA in normal mouse skin. Why a single application of TPA should produce a more prolonged response in initiated mouse skin compared to normal mouse skin is not known. It is obviously an important question to answer. We can say that it is probably not due to a greater and more prolonged proliferative response on the part of the epidermis in initiated mouse skin, since a comparison of the kinetics of epidermal mitotic activity in normal [10] and initiated mouse skin treated with TPA seem to be similar.

Another new finding for TPA induced epidermal growth in initiated mouse skin is the presence of a small transient epidermal hyperplasia within hours after TPA treatment. This transient hyperplasia appears to be what earlier investigators have called an “abortive hyperplasia” [11]. It is of much greater magnitude and is much more dramatic after the application of carcinogens at sufficiently high doses that result in marked epidermal degeneration prior to the second and more lasting hyperplasia [11-13]. “Abortive hyperplasia” is also seen after x-irradiation [14]. Beta-irradiation [15] or alpha-irradiation [16]. In these cases it may last for 7-14 days prior to epidermal degeneration and the onset of the subsequent principal or stable hyperplasia (for recent discussion, see reference 12). It is interesting to note that the transient hyperplasia and the subsequent epidermal degeneration we see after TPA treatment of initiated mouse skin is too small to be reflected in the overall growth kinetics of the epidermis, as measured by changes in epidermal wet weight, total protein, or total DNA. When the transient hyperplasia and the ensuing epidermal degeneration are large enough, as after treatment with 4 nmol esterified cholesterol, they are detected by appropriate changes in epidermal wet weight, protein, and DNA [13,17].

In our earlier investigation of the effects of a single application of 17 nmols of TPA on the skin of normal mice we had not studied the changes prior to day 1 [10]. After completing the reported experiments in this paper, we wondered if treatment of normal mouse skin with TPA also results in a small transient hyperplasia. We have investigated the early changes in normal skin after TPA treatment, and we have found a similar transient hyperplasia at 3-5 hr after TPA application (not shown). The exact relationship of the transient hyperplasia to the accompanying damage, and to the ultimate production of the principal hyperplasia is not known, no matter whether the result of the application of TPA, carcinogens, or radiation. It deserves serious investigation, as we have already noted [12].

Concurrent with the epidermal growth produced by TPA in initiated mouse skin is the presence of considerable epidermal damage. This is also a feature of TPA induced epidermal growth in normal mouse skin [10]. Epidermal damage, after TPA treatment in initiated mouse skin is obvious histologically within hours after TPA application. Thus, it is probably safe to assume it must have begun very soon after TPA application. Consistent with this assumption is the fact that the number of basal epidermal nuclei/mm IFE significantly decreases within 5 hr after TPA application. Although a decrease in basal epidermal nuclei/mm IFE could be due to enlargement of the basal cells, as well as to a more rapid movement of the basal cells into the suprabasal layers, it is likely that at least a portion of the decrease in the number of epidermal/mm IFE is due to cell death. However, one should not conclude that subsequent applications of TPA, as is required for the promotion of tumors, produce the degree of damage that the first application does. Although, the effects of subsequent applications of TPA have not been studied in sufficient detail in either normal or initiated mouse skin, our preliminary studies suggest that the amount of damage produced by chronic administration of TPA is reduced, indicating that perhaps the skin develops a “resistance” to the damaging effects of TPA. The observation that chronic treatment of the skin with TPA or other chemicals, which initially cause considerable damage, result in the development of “resistance” to the skin to these chemicals, is a notion that has been suggested previously by others [18-20].

The fact that there is epidermal damage early after the application of TPA in initiated mouse skin raises the question if the principal epidermal hyperplasia is, in fact, a regenerative hyperplasia. We raised this question in our earlier paper [10] in which we investigated the kinetics of epidermal growth in normal mouse skin following TPA application, where we also saw epidermal damage soon after TPA treatment. The available information is insufficient to allow us to decide whether the epidermal hyperplasia produced by TPA in initiated mouse skin is a regenerative hyperplasia resulting from the epidermal damage produced by TPA. It certainly appears to be the simplest explanation, but this is not sufficient reason to conclude that it is a regenerative hyperplasia. That TPA induced hyperplasia might be a regenerative hyperplasia is suggested by the demonstration that repeated full thickness wounds, or repeated abrasion of initiated mouse skin, which results in epidermal regeneration promotes the appearance of epidermal tumors [21-23]. But, if the damage produced by TPA is the cause of the epidermal hyperplasia, then why are substances such as acetic acid, which is known to have cytotoxic effects on the epidermis, and also results in epidermal hyperplasia, such poor promoters [24]? Clearly, an open mind is required at the moment along with intensive investigation of this intriguing problem.

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REFERENCES

**Announcement**

At the 11th Annual Scientific Meeting of the E.S.D.R. held at the Leeuwenhorst Congress Centre, Nordwijkerhout, 24-27 May, 1981, the following Poster Awards were made.

1. Friends of Bill Reed Committee ($800) to: "Binding Profiles of Lectins to epidermal Langerhans cells." G. Schuler, J. Linert, E. M. Shevach, G. Stingl. Department of Dermatology, Innsbruck Medical School Austria, and NIAID, NIH, Bethesda, MD, U.S.A.