Induction of the Formation of New Hair Follicles in Mouse Tail Epidermis by the Tumor Promoter 12-O-Tetradecanoylphorbol-13-acetate

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SUMMARY

The formation of new hair follicles was quantitatively demonstrated in the tail skin of adult mice in the course of a two-stage carcinogenesis experiment with 7,12-dimethylbenz(a)anthracene as an initiator and the phorbol ester 12-O-tetradecanoylphorbol-13-acetate as a promoter, as well as in experiments with 12-O-tetradecanoylphorbol-13-acetate alone. Two kinds of follicular neogenesis could be distinguished. The most frequently encountered type was characterized by the organization of new follicles from the upper neck and orifice regions of already existing follicles. During their development, these new follicles remained in close apposition to the original follicles but, after having reached a critical size, split off to form fully independent follicles. In the second type of follicular neogenesis, which occurred very rarely, the new follicles seemed to arise directly from the epidermis between two sets of hair triads; however, these follicles never reached their final stage and did not produce hairs.

The formation of new hair follicles may be explained by a "dedifferentiation" of epidermal cells caused by the tumor promoter. Because of the paucity and advanced stage of these follicles, it was possible to verify their relationship to the papillomas by a two-stage carcinogenesis experiment using mouse tail skin. The epidermis in mouse tail skin is much thicker than in back skin and shows a peculiar pattern in that parallel rows of scale-like structures sharply alternate with interscale regions. Keratinization is completely different in these 2 regions; the scales show a parakeratotic type of keratinization, whereas in the interscale zones orthokeratinization with a pronounced granular layer occurs. Groups of 3 hairs are usually localized under each scale. The hairs penetrate the epidermis behind the scales in the interscale fold. This highly regular arrangement of hairs allows the exact quantitation of hairs in a given area of tail skin. In this paper we report the formation of new, independent, hair-producing follicles in mouse tail skin after long-term treatment with the tumor promoter TPA.

INTRODUCTION

A large body of research deals with the role of hair follicles in skin carcinogenesis (1, 3, 12, 16, 22), and there is every reason to believe that a close relationship exists between skin appendages and the development of tumors of the skin (12, 16, 34). In light of the 2-stage carcinogenesis experiment in mouse back skin with DMBA as an initiator and the phorbol ester TPA as a promoter (15), K. Goerttler (personal communication) suggested that the developing papillomas and carcinomas may arise from the hair follicles. The same author claimed to have observed the formation of new hair follicles after long-term promotion in the treated area (13). However, since the density of hairs in mouse back skin is very high, it is almost impossible by histological means to verify a possible relationship between these 2 processes.

The hair frequency per skin area in the mouse tail is considerably lower than that in the back. Moreover, the hairs in the tail are distributed in a highly regular manner. We therefore decided to reproduce the 2-stage carcinogenesis experiment using mouse tail skin.

Female NMRI mice, 7 weeks old, were used in the 2-stage carcinogenesis experiment. The animals were kept under an artificial day-night rhythm as described previously (25). Two groups, each consisting of 28 animals, received an application of 100 nmoles of DMBA in 100 μl of acetone on the whole tail skin, starting 1 cm caudal to the termination of the dorsal pelage. One week later, 20 nmoles of TPA in 100 μl of acetone were applied to the same area of tail skin of one group, whereas the control group received only 100 μl of acetone. This treatment was repeated twice per week over a period of 30 weeks. After 11 and 25 weeks of promotion, 4 mice from each group were sacrificed, and then after 30 weeks 6 mice from each group were sacrificed. The tail skin was examined histologically. The remaining 14 animals of both groups were killed, and the tails were cut off. All tails had been depilated with Pilca cream (Olivin, Wiesbaden, Germany) prior to sacrifice. The whole tail skin was stripped off from bone and cartilage and soaked...
overnight in 1% acetic acid at 4° (9, 31); subsequently, the epidermis was separated from the dermis with a forceps. Two circular sections were punched from each epidermal sheet, stained free-floating in hematoxylin (31), and mounted flat in glycerin jelly. Scales per section were counted under a microscope and the number of scales associated with 2, 3, 4, 5, and 6 hair follicles was determined. Independent of this experiment, the distribution of hair follicle sequences in a given area of tail skin was determined in the depilated tail epidermis of 15-day-old and 7-week-old untreated mice.

RESULTS

Formation of New Hair Follicles. The histological appearance of tail skin of both 15-day-old and 7-week-old mice has already been reported in detail (31). Eleven weeks after topical treatment of tail skin initiated with TPA, the scale region of the epidermis and the interscale region of the epidermis, the latter being normally thinner than the scale region epidermis (31), had thickened considerably as compared with the acetone-treated control animals (not shown). No further significant increase in epidermal thickness was seen after more extended periods (up to 30 weeks) of TPA application (Table 1). The normal scale-interscale pattern with its characteristic features remained unchanged during the entire period of observation, apart from a slight tendency of the interscale granular layer to extend somewhat more into the scale regions.

The method used to separate the epidermis from the dermis caused a rupture of the depilated follicles at the level of the paired sebaceous glands which were firmly anchored in the dermis. Therefore, only the follicular necks were visible in the depilated epidermal sheets (Fig. 2). Upon separation in nondepilated skin, occasionally the entire, hair-containing follicles remained connected to the epidermal sheets. Again, the sebaceous glands were left in the dermis (Fig. 1).

Counting the follicles in normal mice of different ages revealed that sequences of more than 3 hairs per scale were essentially absent from 15-day-old mice, whereas in 7-week-old mice occasionally 4 hairs per scale were noted (Table 1). In the 37-week-old control mice from the 2-stage carcinogenesis experiment, only 1.5% of the scales contained 4 follicles, and scales showing 5 follicles were extremely rare (Table 1). On the contrary, sequences of 4 and 5 follicles per scale and skin area had increased considerably at the expense of the triads in TPA-treated skin (Table 1; Fig. 3). Preliminary results showed that initiation with DMBA was not a prerequisite for the observed alterations. In a study with groups of 6 animals, initiation with DMBA was omitted and TPA was administered according to the application method used in the 2-stage carcinogenesis experiment. A count of the follicles revealed a pattern of distribution comparable to that observed in the 2-stage experiment (control: 982 scales ≤ 3 hairs, 18 scales > 3 hairs; TPA: 939 scales ≤ 3 hairs, 61 scales > 3 hairs; p = 0.005) (c.f. Table 1).

The occurrence of the new follicles was strictly confined to the interscale fold behind the scales and was not observed in the scale regions or in the zones between adjacent scales within 1 ring.

Two kinds of new follicle formation could be clearly distinguished. The most frequently encountered started in close apposition to the upper neck and orifice regions of existing follicles where accumulations of strongly basophilic cells were gradually transformed into follicular buds (Fig. 4). The developing follicle still seemed to be in close contact with the original follicle (Figs. 5 and 6), but the hairs produced in the new follicles clearly emerged from independent piliary canals and orifices (Fig. 7), differing fundamentally from the normal hair replacement mechanism (Figs. 8 and 11). Once the new follicles reached a critical size, they presumably lost their contact with the neighboring follicle and, migrating sideways, formed completely independent hair follicles (Fig. 12).

The newly formed follicles can easily be distinguished from the original ones. The new follicles, due to the limited space under the scale, were squeezed crosswise into the scale troughs, whereas the triad follicles were arranged more or less parallel under the scale (Fig. 1). In most

| Group | Animals | 2 hairs | 3 hairs | 4 hairs | 5 hairs | 6 hairs | Scales associated with
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<tr>
<td>1</td>
<td>15-day-old mice</td>
<td>4</td>
<td>993</td>
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<td>34.62 ± 3.21</td>
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<tr>
<td>2</td>
<td>7-wk-old mice</td>
<td>8</td>
<td>983</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>39.35 ± 2.66</td>
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<td>3</td>
<td>2-stage experiment: 37-wk-old control mice</td>
<td>4</td>
<td>980</td>
<td>15</td>
<td>1</td>
<td>0</td>
<td>43.83 ± 3.21</td>
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<tr>
<td>4</td>
<td>2-stage experiment: 37-wk-old TPA-treated mice</td>
<td>9</td>
<td>930</td>
<td>42</td>
<td>18</td>
<td>1</td>
<td>81.26 ± 9.34</td>
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Table 1

**Determination of the pattern of hair follicle distribution in mouse tail epidermis**

In Groups 1 and 2, 10 circular punch sections (Group 1, r = 0.25 cm; Group 2, r = 0.5 cm) from 10 animals per group were analyzed. In Groups 3 and 4, 28 circular punch sections (r = 0.5 cm) from 14 animals per group were analyzed. Since the absolute number of scales per circular section differed from section to section, the mean values of hair sequences per section were calculated for 1000 scales. Statistical significance of the differences in pattern of hair distribution in Groups 3 and 4 was calculated by a bivariant Wilcoxon test (p = 0.0005). Epidermal thickness was determined in the midscale region under a microscope with a calibrated eyepiece reticule. Values indicated represent the mean value of 20 measurements [4 animals; 4 vertical sections (5 µm); 5 measurements/section]. For values for Groups 1 and 2, see Ref. 30.
cases, the newly formed follicles were derived from the lateral follicles of a triad. A 2nd kind of follicular neoformation was only sporadically encountered. In this case, new follicles did not seem to arise from the neck and orifice regions of original follicles but rather directly from the interfollicular epidermis between 2 sets of triads in 1 scale ring. However, follicles formed in these regions never seemed to reach a normal size, and formation of hairs here was not observed (Figs. 9 and 10).

Formation of Papillomas. Compared with the 2-stage carcinogenesis experiment in back skin of the same mouse strain (15), papillomas arose relatively late and only in small numbers in the tail skin. The formation of carcinomas was not observed. Eleven weeks after treatment, the 1st papilloma was detected in the tail of a mouse. The mice killed at 11 and 25 weeks were without papillomas. From the 20 mice still alive at Week 30 in the TPA-treated group, 5 had developed 1 papilloma and 1 had developed 2 papillomas. These papilloma-bearing mice were killed and the tail skin was examined histologically. None of the mice in the control group developed papillomas.

DISCUSSION

Contrary to the general view that formation of new hair follicles does not occur in mammals after the adult complement has been established, a small but significant increase in hair number was observed in the tail skin of growing mice. This phenomenon was evidenced by the age-dependent appearance of sequences of 4 or more hairs under a scale, where only 3 hairs are normally encountered. Because of the rarity of this event, it is not possible to draw any reliable conclusions as to the mechanism of this kind of hair neogenesis (for instance, a possible hormonal influence). The fact that this natural, although low, tendency was remarkably enhanced by TPA, regardless of whether or not the skin had been treated with a subthreshold dose of DMBA, is important in several respects.

Due to the highly regular distribution of hairs in the form of adjacent triads in the mouse tail, for the 1st time the question whether hair follicles in healing full-thickness wounds arise directly from the covering epithelium (neof ormation) or from viable fragments within this epithelium (regeneration) cannot yet be answered definitively.

It has also been suggested that neof ormation of hairs is associated with certain inflammatory dermatoses as well as epithelial and nonepithelial tumors in humans (26). Furthermore, prodigious numbers of new hairs were produced in the “velvet” covering the annually growing antlers of deer (5). However, this phenomenon cannot be considered only in terms of hair neogenesis, since during this naturally occurring event an entirely new skin, together with its appendages, was formed. Lyne (23) has observed the growth of new hair follicles at the end of each hair cycle in the adult bandicoot (P arameles nasuta Geoffroy), an Australian marsupial. This is the only example of hair neogenesis in an otherwise normal and undamaged adult skin. The process is characterized by the outgrowth of a follicular bud from the outer root sheath of an already existing follicle below the level of the sebaceous glands. The new follicle never separates from its parent follicle, and the hair produced in it penetrates the epidermis through the same pilar canal as does the one that grows in the parent follicle. Since this event can occur several times, bundles of follicles that contain numerous hairs emerging through the same orifice at the epidermal surface are eventually formed (23). Neof ormation of hair follicles by lateral branching from existing follicles has also been reported to occur occasionally at the wound margins of deep wounds in adult sheep (24). In this case, branching took place above the level of sebaceous glands at the follicular neck region, thus closely resembling the type of formation of new follicles in the adult mouse tail after prolonged TPA treatment. However, a subsequent splitting of the newly formed follicles from the parent follicles, as observed in our experiment, was not reported by the other investigators (24).

Most probably, the observed increase in hair number after long-term treatment of tail skin with TPA reflects a true neof ormation of hair-producing follicles, which according to our present knowledge necessarily implies the induction of a dermal papilla. That this is possible in principle has been observed during the process of the formation of bundle hairs (23), where each of the newly formed follicles is equipped with a dermal papilla. As an alternative explanation a TPA-induced stimulation of latent hair anlagen may be assumed which, in turn, would not correspond to true hair neogenesis. However, this can hardly be reconciled with the predominantly observed outgrowth of new hair follicles in close apposition to already existing follicles.

The hair neogenesis in TPA-treated epidermis may be
esters with the desired properties are now in progress in
since in the long-term experiment it caused marked thin
duce new hair follicles, it should be excluded as a control
which has been reported to be a hyperplasiogenic but
this is true, the ability to induce new hair follicles should
ations. Preliminary experiments with ethylphenybpropiobate,
tumor are due to a ‘‘metaplasiogenic” potency of TPA. If
be restricted to tumor-promoting agents and manipula
ments of adult mice of 2 proteins that are normally not detectable
in adult mouse back epidermis but abundantly present in
embryonic or neonatal epidermis of the same body site. Furthermore, it has been shown that the responsiveness of
TPA-treated epidermis to the epidermal G chalone is tran
tiently abolished (21) and that neonatal epidermis does not
respond at all to the G chalone (4). Finally, the same type of
hair neoformation described here in adult mouse tail
skin has been found during the embryonic development of
sheep (8, 11).

It is tempting to speculate that both the formation of a
new organ, i.e., a hair follicle, and the formation of a
tumor are due to a ‘‘metaplasogenic” potency of TPA. If
this is true, the ability to induce new hair follicles should
be restricted to tumor-promoting agents and manipula
tions. Preliminary experiments with ethylphenylpropionate, which has been reported to be a hyperplasogenic but
nonpromoting agent (28), do not allow reliable conclusions.
Although ethylphenylpropionate definitely failed to pro
duce new hair follicles, it should be excluded as a control
since in the long-term experiment it caused marked thin
ning of tail epidermis along with a considerable decrease in
mitotic activity. Experiments with appropriate phorbol esters with the desired properties are now in progress in
our laboratory.

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TPA-induced Hair Neogenesis in Mouse Tail Skin

Fig. 1. Whole mount of epidermis of nondepilatad normal mouse tail, showing follicular triads that are out of phase within 2 adjacent scale rings. Reductions in thickness in the midfollicle regions indicate the site of rupture of the paired sebaceous glands. Hematoxylin. × 80.

Fig. 2. Whole mount of epidermis of depilated tail skin of a 37-week-old control mouse (2-stage carcinogenesis experiment). Note the absence of sequences of 4 follicles under the scales. Hematoxylin, × 40.

Fig. 3. Whole mount of epidermis of depilated tail skin of a 37-week-old TPA-treated mouse (2-stage carcinogenesis experiment) showing an accumulation of sequences of 4 and 5 follicles under the scales. Such a high density of new follicular groups with more than 3 follicles per epidermal area is only occasionally encountered. Hematoxylin, × 60.

Fig. 4. Whole mount of epidermis of depilated tail skin of a 37-week-old TPA-treated mouse (2-stage carcinogenesis experiment). The right follicle of the triad shows a newly forming follicular bud. Hematoxylin, × 400.

Figs. 5 and 6. Whole mount of epidermis of depilated tail skin of a 37-week-old TPA-treated mouse (2-stage carcinogenesis experiment). Parent follicles with closely associated new follicles of advanced stage of development are visible. In Fig. 6, a hair has been retained in the parent follicle. Hematoxylin, × 400.

Fig. 7. Whole mount of epidermis of depilated tail skin of a 37-week-old TPA-treated mouse (2-stage carcinogenesis experiment). Despite depilation, hairs have been retained in the original as well as in the newly formed follicle. Site of ruptured sebaceous glands is clearly visible in both follicles. Hematoxylin, × 400.

Fig. 8. Normal hair replacement showing a growing anagen hair and an old club hair leaving the follicle by the same pilary canal. Epidermal sheet of a 12-week-old normal mouse. Hematoxylin, × 400.

Figs. 9 and 10. Whole mount of epidermis of depilated tail skin of a 37-week-old TPA-treated mouse (2-stage carcinogenesis experiment). Second type of follicular neogenesis. Accumulation of strongly basophilic cells between 2 sets of triads in the epidermal scale fold, leading to a rather rudimentary follicular outgrowth (Fig. 10). Hematoxylin, × 400.

Fig. 11. Transversal section through the whole tail skin of a 12-week-old normal mouse at the level of the sebaceous glands. In the upper triad, a club hair and an anagen hair are localized in the same follicle. Paired sebaceous glands are visible. The different types of keratinization in the scale and interscale regions can be distinguished. H & E, × 300.

Fig. 12. Transversal section through the whole tail skin of a 37-week-old TPA-treated mouse above the level of the sebaceous glands. The new follicle at the left has separated from the original follicle. H & E, × 300.