Changes in Regional Keratin Polypeptide Patterns during Phorbol Ester-mediated Reversible and Permanently Sustained Hyperplasia of Mouse Epidermis

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ABSTRACT

The keratin polypeptide pattern of newborn mouse back, ear, and tail epidermis is uniform and consists of eight individual proteins with a molecular weight range of 46,000 to 67,000. In the adult animal, the keratin patterns of the corresponding body sites are both different from the neonatal pattern and different among themselves. Most notably, ear and tail epidermis contains a high-molecular-weight polypeptide (Mr, 70,000), which is absent from neonatal and adult back epidermis. This postnatal specialization of the local program of keratin polypeptide synthesis cannot be related to distinct morphological criteria.

We have analyzed the behavior of these regional keratinization phenotypes in the adult mouse under the influence of a reversible or a permanently sustained hyperplasia. Both types of epidermal trauma were induced by either single or repeated treatment of skin with tumor-promoting or nonpromoting hyperplasiogens. Independent of the type of hyperplasiogen used, the initial phase of a reversible hyperplasia (0 to 12 hr) is characterized by only marginal morphological alterations, maintenance of the normal one-dimensional keratin pattern, but pronounced changes in the charge properties of distinct keratin polypeptides, indicating disturbances of the secondary phosphorylation-dephosphorylation process. During the acute phase of reversible hyperplasia (1 to 7 days), the local keratinization phenotypes are quantitatively altered due to the enhanced expression of high-molecular-weight keratin polypeptides. However, the charge properties of the newly synthesized proteins appear normal. A comparison of both the sequential morphological alterations during acute hyperplasia and the temporally corresponding changes in the keratin patterns allows an assignment of the site of synthesis of distinct keratin polypeptides within the epidermis, which is in line with findings of other laboratories. Long-term hyperplasia (2 weeks to 2 months of regular treatment) is not morphologically different from maximum reversible hyperplasia. However, it induces both quantitative and qualitative alterations of the local keratinization phenotypes and causes a reversal of the postnatal divergence of keratin synthesis by generally restoring the neonatal keratin pattern in all three adult epidermal sites investigated.

INTRODUCTION

Keratin polypeptides, the constituent proteins of epidermal intermediate-sized filaments (tonofilaments), belong to the class of highly insoluble epidermal components. They represent by far the most abundant differentiation products of the epidermis and as a rule show up electrophoretically as a protein family with a molecular weight range of 40,000 to 70,000. It is now well established that the keratin pattern within different normal epithelia of one species may be subject to considerable alterations (3, 11, 27, 45). Depending on the anatomical region, this protein heterogeneity may be merely quantitative (when differences occur only in the intensity of distinct polypeptides), or there may be also qualitative differences in the number of keratin subunits. The reason for these regional modifications of the keratin pattern, which cannot reliably be related to distinct morphological criteria (3), are unknown. There is, however, evidence from homologous and heterologous epidermaldermal transplantation studies that dermal factors may be involved in the regulation of epidermal differentiation (6, 39, 44).

Alterations in the local keratin polypeptide patterns have furthermore been demonstrated in diseased epidermis of humans (1, 4, 23, 40, 42) and also of laboratory animals (45, 46). Epidermal cells of different origin grown in culture under common conditions respond rather uniformly to the artificial environment in that they no longer synthesize keratin polypeptides above the molecular weight level of 60,000 (12, 14, 22, 41). This phenomenon is, however, fully reversible, and cultured cells again regain their in vitro phenotype when transplanted back into living animals (9, 14). All these findings emphasize the impressive susceptibility of the program of epidermal keratin polypeptide synthesis to endogenous and exogenous changes of normal growth conditions.

In this study, we have investigated the normal keratin polypeptide composition of various body sites of the newborn and adult mouse epidermis and their alterations in relation to the induction of either a reversible or a permanently sustained hyperplasia. To produce both types of hyperplasia, we have used, for several reasons, chemical hyperplasiogens belonging to the family of phorbol esters. On the other hand, some of these natural compounds, e.g., TPA, exhibit besides their hyperplasiogenic effect a strong skin tumor-promoting capacity, whereas only slightly modified structural analogs of TPA act solely as hyperplasiogens (18). Moreover, the morphological (2, 32–34) and molecular effects (for review, see Ref. 8) of these compounds on the epidermis, brought about by lowest, nearly nontoxic doses, have been extensively studied.

Using both 1- and 2-dimensional gel electrophoresis, we describe keratin polypeptide heterogeneity between neonatal...
and adult mouse epidermis and confirm the existence of both site-specific keratin patterns in normal adult epidermis. Independent of a promoting ability of the hyperplasiogen used, we furthermore present evidence that, during a short period after injection of a reversible hyperplasia, considerable deviations of the normal posttranslational protein modification reactions occur and that a long-lasting hyperplasia causes adult tissues to reexpress the neonatal keratin polypeptide pattern.

MATERIALS AND METHODS

Production of Reversible and Permanently Sustained Hyperplasia. A reversible hyperplasia was induced by a single application of 20 nmol of the tumor-promoting phorbol ester TPA in 100 μl of acetone to the carefully shaved back skin or of 10 nmol of TPA in 50 μl of acetone to the ear skin of an adult mouse (strain NMRI, bred at the German Cancer Research Center). Alternatively, the nonpromoting or weakly promoting agents [4-O-methyl-TPA (back skin, 400 nmol; ear skin, 200 nmol) or mezerein (back skin, 10 nmol; ear skin, 5 nmol)] were used to induce reversible hyperplasia in these tissues. The development of the hyperplasias was investigated histologically over a period of 14 days (5-μm sections stained with hematoxylin and eosin).

The permanently sustained hyperplasia of back and ear epidermis was brought about by 3 applications per week of TPA, 4-O-methyl-TPA, or mezerein using one-half of the concentrations indicated above. Duration of treatment was 2 and 4 weeks, respectively. Tail epidermis was treated 3 times weekly with 40 nmol of TPA (800 nmol of 4-O-methyl-TPA) for 2 months or daily with 200 IU of vitamin A acid (Serva, Heidelberg, Germany) in 100 μl of a mixture of 20% propylene glycol-10% Tween-20-30% glycerol-40% water for 2 weeks (38).

In all long-term experiments, the treated skin area was removed for keratin analysis and histological examination (5-μm sections stained with hematoxylin and eosin) 6 and 24 hr after the last application of the corresponding hyperplasiogen.

Extraction of Keratins. A modification of the keratin extraction method described by Franke et al. (10) was used. Back and tail skin of killed adult animals was carefully depilated with Pilca cream (Olivin, Wiesbaden, Germany), whereas ear skin was left without depilation. The epidermis of both newborn and adult mice were generally separated from the dermis by means of the acetic acid method (5) and was carefully ground in a mortar with liquid nitrogen cooling. For removal of soluble epidermal proteins, the fine powder was homogenized in a high-salt buffer (1.5 M KCl-10 mM NaCl-2 mM dithioerythritol-0.5% Triton X-100-10 mM Tris-HCl, pH 8.0) with 20 strokes of a tightly fitting Potter-Elvehjem homogenizer. After centrifugation for 30 min at 15,000 rpm, the pellet was reextracted 5 times with the high-salt buffer. The resulting high-salt insoluble residue was then stirred overnight in 5% SDS-5% β-mercaptoethanol-10 mM Tris-HCl, pH 8.0 (50 μl/10 mg of wet residue), heated for 30 min at 80°, and centrifuged for 1 hr at 15,000 rpm, and the keratin-containing supernatant was removed and stored frozen at −20°.

In Vivo Labeling of Keratins. The back skin of 20 adult mice was carefully shaved, and 1 day later, 4 animals received an s.c. injection of 100 μCi of a 14C-labeled amino acid mixture in 300 μl of 0.9% NaCl solution (L-[U-14C]amino acid mixture, FB 104; 50 μCi/ml; Amersham Buchler, Braunschweig, Germany). The remaining 16 animals were divided into 2 groups, 8 animals being topically treated with 20 nmol TPA and 8 animals receiving 400 nmol 4-O-methyl-TPA each. Subsequently, 4 animals of each group were given injections of the labeled amino acids after 1 hr. The remaining animals were given injections of the labeled amino acids 66 hr after the application of the hyperplasiogen. A group of 10 newborn mice received an s.c. injection of 10 μCi of radioactive amino acids in 100 μl of 0.9% NaCl solution.

In both newborn and adult animals, the injection produced a fairly well visible dorsal blister, the borders of which were outlined with a pen. Six hr after the injection of radioactive amino acids, animals of all groups were killed. The back skin of adult mice was deceptinated, the marked skin areas in newborn and adult mice were excised, and the epidermis was separated from the dermis by means of the acetic acid method. Keratins of the pooled epidermal fractions of all 6 experimental groups were extracted as described above.

Extraction of Epidermal Polyadenyllic Acid-containing mRNA and In Vitro Translation of Keratins. Total cellular RNA of newborn mouse back epidermis was isolated by a one-step procedure using 4 M guanidium thiocyanate homogenization and CsCl centrifugation as described previously (37). Polyadenylated RNA was separated from total cellular RNA by oligo deoxythymidy late-cellulose column chromatography (oligo deoxythymidylate-cellulose, type 7; Collaborative Research, Inc., Waltham, Mass.) and stored frozen at −70°.

Polyadenylate-rich fractions were translated in a commercially available mRNA-dependent cell-free reticulocyte lysate translation system (NEK-001 reticulocyte lysate-[35S]methionine translation kit; New England Nuclear, Boston, Mass.) in the presence of [35S]methionine. Following 1 hr of incubation at 37°, the stimulatory activity of the epidermal mRNA fractions was determined in relation to the endogenous control value according to the translation kit instructions.

Analytical Procedures. One-dimensional SDS-polyacrylamide gel electrophoresis was performed in a slab gel apparatus using the discontinuous system described by Laemmli (24). Protein concentration in the individual keratin probes was determined according to a slightly modified method of Schaffner and Weissmann (35). In general, the gels were loaded with 50 μg of protein per slot. Gels were stained in a solution of 0.1% Coomassie Brilliant Blue R-250 in 25% isopropl alcohol and 10% glacial acetic acid and destained in 7% acetic acid at 60°.

Two-dimensional electrophoresis analysis of keratins was carried out according to the method described by O’Farrell (31) with minor modifications introduced by Garrels and Gibson (15). Electrotocusing was performed in 4% polyacrylamide gels in capillary tubes in the presence of 9.5 M urea, 4% Nonidet P-40, 5% β-mercapto-ethanol and ampholines, pH 5.8, at 500 V for 16 hr. For electrophoresis in the second dimension, the isoelectrically focused gels were equilibrated for 10 min in 3% SDS-50 mM dithioerythritol-62.5 mM Tris-HCl, pH 6.8, and applied to SDS-polyacrylamide gels described above.

Both 1- and 2-dimensional destained gels were subjected to fluorography as described by Bonner and Laskey (7) and autoradiographed at −70° on Kodak X-Omat R films.

RESULTS

Production of Reversible and Permanently Sustained Hyperplasia in Adult Epidermis of Different Body Sites. The morphology of the thin interfollicular back epidermis of the adult mouse is characterized by the almost complete lack of a granular layer and contains at best a barely distinguishable spinous layer. The epithelium consists predominantly, therefore, of the basal and the horny layer (Fig. 1a). Since several detailed light and electron microscopic descriptions of TPA-mediated hyperplasia in mouse back epidermis exist in the literature (2, 32-34), we were able to confine ourselves to a comparative light microscopic observation of the time course of onset, peak, and decline of the reversible back epidermis hyperplasia. Within 16 hr of the application of a tumor-promoting dose of TPA (20 nmol), there is a measurable increase in the thickening of the interfollicular epidermis which is exclusively due to a generalized hypertrophy of cells of all layers and a widening of the intercellular space (2, 32).

Owing to the dramatic increase in the proliferation of basal cells between 24 and 40 hr (22, 32, 34), a marked acanthosis develops which is followed at about 40 hr by the formation of a pronounced...
granular layer. Due to the subsequent strong hyperkeratosis, the epidermal hyperplasia reaches its maximum between 48 and 96 hr and consists now of 5 to 7 living cell layers (Fig. 1d) (2, 32). During that time, the hyperplastic epidermis resembles very much the neonatal epidermis. From Day 5 onwards, the hyperplasia gradually declines at the expense of the spinous layer, with concomitant preservation of a strong hyperkeratosis. The epidermal morphology is again back to normal 10 to 12 days after the application of TPA (33).

An almost identical time course and degree of the hyperplastic process are observed after a single application of 10 nmol TPA to ear epidermis which, like back epidermis, represents a very thin epithelium containing, however, a histologically distinguishable stratum spinosum (38).

In both back (13) and ear epidermis, a hyperplasia comparable to that produced by the tumor promoter TPA can be induced by means of appropriate doses of the nonpromoting phorbol ester 4-O-methyl-TPA or the weak promoter mezerein (Ref. 20; Footnote 3).

Long-term application of TPA, 4-O-methyl-TPA, or mezerein to both back and ear epidermis using a 48-hr interval administration schedule leads to the establishment of a stationary hyperplasia of the tissue (17) which morphologically resembles closely the maximum hyperplasia seen after single application of the hyperplasiogens.

Adult mouse tail epidermis (Fig. 1c) represents one of the most unusual epithelia of the mouse, in that it is build up by regularly alternating sequences of parakeratotic scale regions and intervening orthokeratotic interscale regions (19, 38). Long-term application of TPA or 4-O-methyl-TPA results in a strong hyperplasia of both epidermal regions without loss of characteristic para- and orthokeratotic patterning (Fig. 1d). In contrast, a daily treatment of tail skin over 2 weeks with low doses of antipromoting vitamin A acid (43) leads to the induction of orthokeratosis in the scale regions, so that the entire strongly hyperplastic tissue possesses a continuous granular layer (Fig. 1e) (19, 38).

One- and 2-Dimensional Keratin Polypeptide Patterns of Different Keratinizing Epithelia of the Neonatal and Adult Mouse. To examine the keratin composition in epithelia of different anatomical localizations, epidermis was separated from the back, ear, and tail skin of newborn and adult mice. To avoid contamination with residual hair shafts, back and tail skin of adult mice was depilated prior to tissue separation. Histological examination of the depilated tissues did not reveal any loss of cornified layers. Keratins were extracted with an SDS-/S-mercaptoethanol buffer after extensive removal of soluble epidermal proteins with a buffer of high ionic strength (46).

In Fig. 2, Slot A, the Coomassie-stained keratin polypeptides of newborn mouse epidermis are shown. The keratin sample originates from ear epidermis, but virtually identical patterns are encountered in back (Fig. 5, Slot A) and tail epidermis (Fig. 7, Slot L). Therefore, independent of the body site, newborn mouse keratin consists of 8 individual proteins with a molecular weight range of 46,000 to 67,000 (see legend to Fig. 2). By intensity, polypeptides 5 and 6 are the most abundant components of the keratin family, whereas polypeptides 2 to 4 are present in lower, but nearly identical, concentrations. In contrast, the low-molecular-weight components 7 to 9 are only faintly expressed, protein 8 being detectable only in heavily overloaded gels (see Fig. 4c, 1-dimensional inset). Of these proteins, normal adult back epidermis (Fig. 2, Slot B) contains proteins 5, 6, and 7 as the most prominent bands. In contrast, both ear and tail epidermis (Fig. 2, Slots C and D) are qualitatively different from these 2 patterns. Most notably, they contain an additional high-molecular-weight protein 1 (M, 70,000), present in comparable amounts in both tissues. Unlike adult mouse back epidermis, protein 2 is also a major constituent of the keratin family of ear and tail epidermis. The intermediate proteins 3 and 4, as well as the low-molecular-weight component 7, are far more pronounced in tail epidermis than in ear epidermis. In most cases, a slight contamination of the keratin preparations with actin could not be avoided.

When investigated 2-dimensionally, it is a general observation that the individual members of the keratin family exhibit a pronounced but tissue-independent charge heterogeneity (Fig. 3). This gives rise to a staggered pattern of the keratin polypeptides from acidic to basic pH values, varying according to the size of the polypeptides. This particular positioning of keratin polypeptides with regard to charge and size could also be demonstrated in keratinizing epithelia of humans (41), rats (16, 45), and hamsters (45).

The ubiquitous proteins 5 and 6 are mainly resolved in 3 to 4 closely spaced spots with pl values in the range of 5.3 to 5.4 (Fig. 3, a to c). This also corresponds to the pH range within which the small proteins 7 and 8 are focused, whereas the smallest polypeptide 9 (as the most acidic member of the keratin family) is resolved into 2 spots between pl 5.0 and 5.2 (Fig. 3b).

If present, the intermediate proteins 3 and 4 show extensive charge heterogeneity on the more basic side of the gels over a relatively wide pH range of 5.9 to 7.5 (Fig. 3, a and c). These proteins are not always resolved into distinct spots and are sometimes present as streaks (Fig. 3a). Tailing of high-molecular-weight components has similarly been observed in human keratins (41). As a rule, the largest components, proteins 1 and 2, do not enter the focusing gel in the given pH range, but protein 2 is sometimes detectable at the alkaline borderline of the gels.

Early Effects (0 to 14 Hr) of a Reversible Hyperplasia on the Keratin Pattern of Back Epidermis. During the first 14 hr after induction of a reversible hyperplasia by a single topical application of TPA, the normal keratin polypeptide pattern of back epidermis undergoes no detectable changes when investigated 1 dimensionally at 1-hr intervals (results not shown). On the other hand, substantial deviations from the normal focusing pattern can be detected during this period on 2-dimensional gels (Fig. 4). At the height of keratin polypeptides 5 and 6, horizontal lines of multiple, closely spaced spots appear between pl 5.6 and 6.5 outside the normal focusing range in the more alkaline part of the gels, the protein moiety at the height of polypeptide 5 migrating consistently further into the alkaline region. A similar, although less pronounced, effect is seen at the height of protein 7 (Fig. 4c). Conversely, a shift in the opposite direction is revealed at the molecular weight level of the contaminating actin component (Fig. 4, b and c). Whereas only a very faint spot appears at the normal actin position (pl 5.5), 2 well-defined spots show up in the most acidic part of the gel (pl 5.0 to 5.1).

These disturbances in the charge properties of the proteins,
already visible 1 hr after the induction of the hyperplasia (Fig. 4d), persist up to 12 hr, the pattern after 14 hr being almost normal with regard to the positioning of the keratin polypeptides (Fig. 4d). The same phenomena can be observed after induction of a reversible hyperplasia of back epidermis with 4-O-methyl-TPA or mezerein (not shown).

Late Effects (1 Day to 14 Days) of a Reversible Hyperplasia on the Keratin Patterns of Back and Ear Epidermis. One day after a single treatment of back skin with TPA, protein 2 in addition to the usual keratin polypeptides 5, 6, and 7 appears as a strong band on 1-dimensional gels (Fig. 5, Slot C). This pattern occurs during the time of formation of the stratum spinosum hyperplasia (Fig. 5, Slots C and D). Concomitant with the onset of enhanced keratinization is the appearance of the intermediate proteins 3 and 4 (Fig. 5, Slot G). In accordance with the morphological similarities within this period, the keratin pattern of hyperplastic back epidermis is almost identical to that of neonatal epidermis (Fig. 5, Slot A). Again, as with the morphological features, 14 days after induction of the hyperplasia, the keratin polypeptide pattern is back to normal (Fig. 5, Slot H).

As already indicated by the 14-hr 2-dimensional keratin pattern (Fig. 4d), the keratin polypeptides also investigated during the subsequent period of acute hyperplasia do not show deviations from their charge properties (Fig. 4e). As in normal tissue, the newly synthesized polypeptides 3 and 4 are resolved into multiple spots within an pI range of 5.6 to 6.5.

Despite the striking similarities in morphology of the TPA-induced hyperplasia of area ear and back epidermis, the changes at the keratin polypeptide level are much less pronounced in hyperplastic ear epidermis (Fig. 6). The intensity of both protein 1 and protein 2 is not visibly affected during the whole period of hyperplasia, and the only detectable alterations are restricted to proteins 3 and 4. As in back epidermis, their synthesis is enhanced during the period of epidermal hyperkeratosis (Fig. 6, Slots E and F). At no time point, therefore, did the keratin pattern in reversibly hyperplastic ear epidermis resemble that of neonatal epidermis.

As in back epidermis, the keratin patterns of ear epidermis did not show alterations in 2-dimensional gels of the position of the individual polypeptides during acute hyperplasia (Fig. 4f). Furthermore, the observed late effects in both tissues were again independent of the tumor-promoting properties of the hyperplasiogens used.

Effects of a Permanently Sustained Hyperplasia on the Keratin Patterns of Adult Mouse Back, Ear, and Tail Epidermis. As shown in Fig. 7, the keratin polypeptide pattern of permanently stimulated (2 weeks) back epidermis is qualitatively not different from that seen during maximal hyperplasia after a single application of the hyperplasiogen (Fig. 7, Slots B and C), both patterns resembling the neonatal pattern in Slot L.

A substantially different situation is encountered in ear epidermis. Whereas the keratin pattern of a reversible hyperplasia at a maximum does not correspond to the neonatal pattern (Fig. 7, Slot E), a 2-week-long permanent hyperplasia is sufficient to cause a complete loss of polypeptide 1, thus restoring the neonatal pattern in ear epidermis (Fig. 7, Slot F). The same phenomenon could be observed after long-term treatment of ear skin with 4-O-methyl-TPA (not shown) or mezerein (Fig. 7, Slot G).

Unlike ear epidermis, a sustained TPA hyperplasia (2 months) in tail epidermis, which leaves the particular morphology of this tissue intact (Fig. 1d), can at most be interpreted as a step towards restoring the neonatal keratin pattern, since protein 1, although drastically reduced in intensity, is still present in the keratin pattern (Fig. 7, Slot I). Again, the non-promoting 4-O-methyl-TPA was found to produce identical morphological and biochemical alterations (Fig. 7, Slot J). In contrast, vitamin A-mediated sustained hyperplasia not only leads to a uniformly orthokeratinizing tissue (Fig. 1e) but also to the reexpression of the neonatal pattern in tail epidermis (Fig. 7, Slot K).

As a general observation in permanently sustained hyperplasia, the enhanced synthesis of the low-molecular-weight keratin polypeptides 8 and 9 should be emphasized.

None of the keratin preparations from long-term-treated epidermis showed deviations from the normal positioning of the polypeptides in 2-dimensional gels, including those which were isolated shortly (6 hr) after the last application of the hyperplasiogen.

In Vivo Labeling and In Vitro Translation of Keratins. Injections of 100 μCi of 14C-labeled amino acids into the back skin of untreated mice did not lead to a detectable incorporation of label into keratin polypeptides within 6 hr. The same negative result was obtained when the animals were exposed to the label 1 hr after TPA treatment. In both experiments, incorporation of radioactive amino acids was found mainly in the fraction of soluble epidermal proteins (results not shown).

Sixty-six hr after TPA application, the strongly hyperplastic epidermis is roughly at the transition from the proliferating to the differentiating phase of hyperplasia. Injection s.c. of 100 μCi of radioactive amino acids for 6 hr at this time point leads to an incorporation of label into all keratin polypeptides (Fig. 8, Slot D) demonstrable also by protein staining (Fig. 5, Slot E), the ratio of radioactivity to protein, as estimated visually from the autoradiogram (Fig. 8, Slot D) and the Coomassie-stained pattern (Fig. 5, Slot E), being almost identical for each polypeptide.

Parallel to the finding that Coomassie-stained 2-dimensionally resolved keratin polypeptides extracted from epidermis during acute hyperplasia did not show deviations from the normal focusing pattern (Fig. 4, e and f), the autoradiogram of the newly synthesized labeled keratins revealed a normal 2-dimensional distribution of the proteins (Fig. 9a).

Newborn mouse epidermis, having a 3-fold-higher proliferation rate than adult back epidermis, also incorporates label into all keratin polypeptides identifiable by protein staining (cf. Fig. 8, Slot C, and Fig. 2, Slot A). There is, however, a remarkable discrepancy in the ratio of radioactivity to protein with regard to the intermediate proteins 3 and 4. When analyzed 2-dimensionally, the keratin polypeptides and the contaminating actin component, synthesized de novo in a rapidly proliferating tissue, give the normal focusing pattern (Fig. 9b).

A similar discrepancy in the protein and labeling intensities of proteins 3 and 4 is seen in 1-dimensional gels of keratins translated in vitro from mRNA of newborn mouse epidermis (cf. Fig. 8, Slots A and B, and Fig. 2, Slot A). In accordance with previous findings, actin is a major in vitro translation product outside the keratin range (12, 37).
Surprisingly, the 2-dimensionally resolved in vitro translation products from mRNA of newborn mouse epidermis show a similar charge heterogeneity at the molecular weight level of keratin polypeptides 5 and 6, as is observed during the early phase of a reversible hyperplasia of adult epidermis (Fig. 9, c and d). Furthermore, also, the actin component gives rise to spots migrating to lower pl values, although this tendency is less pronounced than in the in vivo experiments.

DISCUSSION

This investigation reveals that the regional diversity in the keratin composition within one species seems to be especially apparent in the adult mouse, which has quantitatively and qualitatively different keratin patterns in epithelia of the back, ear, and tail, in the footpad epidermis, and in the keratinizing vaginal and forestomach epithelium (45). However, as with previous findings, these numerous examples of local keratin heterogeneity do not offer reliable clues as to a possible correlation of epidermal morphology with keratin patterns.

Each of these adult keratin variants develops from a uniform neonatal keratin pattern not yet subject to regional modifications, the individual polypeptides being progressively synthesized during embryogenesis. Since recent experiments have provided evidence that each individual keratin polypeptide is encoded by its own mRNA (12) and that the qualitatively different keratin patterns of neonatal mouse epidermis and adult mouse tail epidermis are faithfully translated in vitro from mRNA preparations of the corresponding tissues (37), the postnatal keratin modifications are therefore genetically programmed, and local keratinization phenotypes may be said to exist.

If epidermis is hyperplastically transformed, these regional keratinization phenotypes react sensitively but not uniformly to the reversible hyperplasia, whereas a permanently sustained hyperplasia invariably gives rise to a more uniform response.

The particularly thin back epidermis of the adult mouse is ideally suited for studies of this type. In connection with the promotion phase of the 2-stage skin carcinogenesis experiment, almost exclusively performed at this body site, not only the sequential morphological alterations (2, 32–34) but also the complex biochemical response of the epidermis to short-term and long-term regimen of both tumor-promoting and non-promoting hyperplasiogens have been analyzed extensively (for review, see Ref. 8).

According to these investigations, the initial phase of a reversible hyperplasia is characterized by not only marginal morphological changes of the tissue but also a period of dramatic molecular alterations of a variety of factors involved in the control of epidermal growth and differentiation (for review, see Ref. 8).

The alterations seen at the keratin level during this early phase of hyperplasia fit into this general scheme. Whereas the overall keratin polypeptide composition remains stable, considerable changes occur in regard to the charge properties of the proteins, in that almost instantly after the hyperplasiogenic impact, protein moieties at the molecular weight level of acidic keratin members appear in the less acidic region of the gels.

The resolution of these abnormally focused protein moieties into multiple spots with constant interval distances points to a regularly repeated alteration of the molecules.

All of the intermediate-sized filaments known have been shown to possess phosphorylated subunits (25). This post-translational modification is brought about by cyclic adenosine 3':5'-monophosphate-dependent protein kinases (26), the amino acid serine being the phosphate acceptor (16, 30). Each of the keratin polypeptides extractable from adult rat back epidermis (the keratin pattern of which is fairly identical to that of neonatal mouse epidermis in number and size distribution) is heavily phosphorylated in vivo within 15 min (16). Recently, Link and Marks (28) were able to demonstrate the existence in mouse back epidermis of a complete phosphorylation-dephosphorylation system. The early observable TPA-induced charge heterogeneity at the molecular weight level of mouse keratin polypeptides could therefore be attributed to incomplete secondary phosphorylation.

Alternatively, a TPA-induced enhanced protein dephosphorylation reaction may be responsible for the observed shift of protein moieties into more alkaline regions. The rather surprising finding that keratin polypeptides translated in vitro from mRNA of normal tissue display a charge heterogeneity comparable to that seen in vivo shortly after TPA treatment is not easy to evaluate. Since protein kinases and phosphoprotein phosphatases are ubiquitous enzymes, their presence in the crude reticulocyte translation lysate is conceivable, and it may be their ratio which determines the ability of the artificial system to faithfully reproduce the tissue-specific in vivo mechanisms of protein biosynthesis in all respects.

In view of the fact that the type of TPA-induced reversible hyperplasia represents the most common benign injury of epidermis, it is rather self evident that the tissue is programmed for a rapid repair of the impairment of the biosynthesis of its differentiation products. Obviously, long before the phase of active de novo synthesis of keratins, the enzyme system responsible for posttranslational protein modification seems to be fully reequilibrated and, as shown by the autoradiographically revealed position of newly synthesized keratin polypeptides, remains undisturbed during the entire period of acute hyperplasia. On the other hand, the capacity of the tissue to overcome the described damage may be continuously affected in pathologically altered epidermis. Recently, we were able to show that, in addition to distinct qualitative alterations of the keratin pattern, perturbations in posttranslational polypeptide modifications may be persisting properties of malignant epidermal tumors (45, 46).

Concomitant with the onset of morphological alterations in TPA-treated back epidermis, notable changes can be detected in the keratin pattern on 1-dimensional gels, in that the expression of the large, normally faint polypeptides is progressively enhanced. A similar quantitative increase of high-molecular weight keratin members in hyperplastic epidermis has been described by Baden et al. (3).

Recent experiments have provided clues as to the relation of the site of synthesis of the particular keratin polypeptides to distinct epidermal layers (11). It appears that basal cells are predominantly responsible for the synthesis of keratin polypeptides up to the molecular weight level of 60,000, whereas members of higher molecular weight are generated by cells of the suprabasal living layers. Both the morphology and the
keratin patterns of all normal adult epithelia of the mouse and, more importantly, the sequence of temporal changes of these criteria in hyperplastically transformed back and ear epidermis corroborate conclusively this assignment of the site of keratin polypeptide synthesis. Owing to the extreme morphological heterogeneity of the adult epidermal tissues which contain the highest-molecular-weight keratin polypeptide 1, and owing to its lack in neonatal epidermis, the site of synthesis of this protein remains obscure. It is noteworthy in this context that polypeptide 1 is typically encountered in integumental body sites with a very low density of hairs (i.e., ear and tail) or in the completely glabrous sole-of-the-foot epidermis.\(^5\) For obvious reasons, a relation to parakeratosis, as deducible from the TPA and vitamin A experiments in tail epidermis, is not tenable. Immunologically oriented investigations may be the only suitable means to overcome this lack of information.

The transient restoration of a qualitatively neonatal keratin pattern in back epidermis treated once with TPA or the permanent reestablishment of pattern in back and ear epidermis under continuous influence of TPA is interesting, since a tumor promoter-specific reprogramming of tissue differentiation (as a prerequisite of the expression of the transformed state) has been discussed in connection with the still enigmatic mechanism of tumor promotion (29). Without precluding such a phenomenon, the present study has revealed, however, that none of the effects on the synthesis of one of the major differentiation products of the epidermis can selectively be related to the promoting property of TPA. This is because the kinetics of appearance and disappearance of keratin polypeptide alterations, in addition to the quantitative and qualitative changes seen in the epidermises of different body sites, can also be brought about by nonpromoting hyperplasiogens. Moreover, as demonstrated by the vitamin A effect on tail epidermis, there are even antipromoting hyperplasiogens displaying a stronger capacity to affect the state of epidermal differentiation.

The tendency of adult mouse epithelia to reverse postnatal specialization of keratin polypeptide synthesis as a consequence of a long-lasting trauma in favor of the reestablishment of neonatal tissue conditions is the most striking finding of our study. Obviously, the time required for this process depends on the degree of postnatal specialization, and its physiological advantage may tentatively be explained by the fact that an optimal repair of tissue damage is best brought about by a transitory repetition of tissue ontogenesis.

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REFERENCES

33. Raick, A. N. Late ultrastructural changes induced by 12-O-tetradecanoylphorbol-13-acetate in mouse epidermis and their reversal. Cancer Res., 33:
34. Raick, A. N., Thumm, K., and Chivers, B. R. Early effects of 12-O-tetrade- 
canoyl-phorbol-13-acetate on the incorporation of tritiated precursor into 
DNA and the thickness of the interfollicular epidermis, and their relation 
35. Schaffner, W., and Weissmann, C. A rapid, sensitive, and specific method 
36. Schweizer, J. Synthesis of histidine-rich proteins in embryonic, adult, and 
stimulated epidermis in different mammals. Front. Matrix Biol., 9: 127–141, 
1981.
37. Schweizer, J., and Goerttler, K. Synthesis in vitro of keratin polypeptides 
directed by mRNA isolated from newborn and adult mouse epidermis. Eur. 
38. Schweizer, J., and Marks, F. A developmental study of the distribution and 
frequency of Langerhans cells in relation to formation of patterning in mouse 
39. Sengel, P. The determinism of the differentiation of the skin and the cuta-
neous appendages of the chick embryo. In: W. Montagna and W. C. Lobitz 
40. Skerrow, D., and Hunter, I. Protein modifications during the keratinization of 
484, 1978.
41. Sun, T-T., and Green, H. Keratin filaments of cultured human epidermal 
42. Thaler, M. P., Fukuyama, K., Inone, N., Cram, D. L., and Epstein, W. L. Two 
Tris-urea-mercaptopropanol extractable polypeptides found uniquely in 
Inhibition of skin tumor promotion by retinoic acid and the metabolite 5,6-
44. Wessels, N. K. Tissue interactions during skin histodifferentiation. Dev. Biol., 
45. Winter, H., and Schweizer, J. Carcinoma-specific keratin polypeptide pat-
terns in keratinizing epithelia of rodents: independence of species and tissue 
46. Winter, H., Schweizer, J., and Goerttler, K. Keratins as markers of malig-

Fig. 1. Normal and hyperplastically transformed epidermis of different anatomical regions of the adult mouse. a, normal back epidermis (depilated). b, back 
epidermis 72 hr after a single topical treatment with 20 nmol TPA. Note the increase in thickness of the extremely thin normal back epidermis into a multi-layered 
epithelium containing well-developed strata of spinous, granular, and horny cells. c, normal tail epidermis. Note the alternating sequences of parakeratotic scale 
regions (ISR) with a densely packed horny layer and the orthokeratotic inter-scale regions (ISR) with a flaky ("basket-weave") horny layer. Both keratinized layers 
stain differently with H & E. d, tail epidermis treated 3 times weekly for 2 months with 40 nmol TPA. The strongly hyperplastic epidermis has retained its characteristic 
ortho- and parakeratotic patterning. Arrows, inter-scale granular layer and the flaky horny layer. e, tail epidermis treated daily for 2 weeks with 200 IU vitamin A acid. 
Note the continuous granular layer and the loosely packed horny layer of the strongly hyperplastic epidermis. All sections were cut at 5 µm. a and b, H & E; × 150; 
c to e, H & E; × 100.
Fig. 2. One-dimensional keratin polypeptide patterns of mouse epidermis. Slot A, newborn mouse ear epidermis; Slot B, adult mouse back epidermis; Slot C, adult mouse ear epidermis; Slot D, adult mouse tail epidermis. Keratin samples were electrophoretically resolved and stained with Coomassie blue on a 9% polyacrylamide gel. Arrowheads indicate the migration distances of bovine serum albumin (M, 68,000) and actin (M, 43,000). The estimated molecular weights of the individual keratin polypeptides are: 1 (M, 70,000); 2 (M, 67,000); 3 (M, 64,000); 4 (M, 62,000); 5 (M, 60,000); 6 (M, 58,000); 7 (M, 52,000); 9 (M, 46,000). Polypeptide 8, visible only on strongly overloaded gels (see 1-dimensional inset in Fig. 4c) has an estimated molecular weight of 49,000.

Fig. 3. Two-dimensional keratin polypeptide patterns of mouse epidermis. The keratin samples were analyzed by isoelectric focusing in the first dimension (IEF; pH gradients 5 to 8) and SDS-polyacrylamide gel electrophoresis in the second dimension (SDS; 8.7% gels). Numbering of spots according to molecular weight corresponds to that of the 1-dimensionally resolved keratin polypeptides in Fig. 2. a, newborn mouse epidermis; b, adult mouse back epidermis; c, adult mouse tail epidermis.
Keratins and Epidermal Hyperplasia

Fig. 4. Effect of reversible hyperplasia on the 2-dimensional keratin polypeptide pattern of adult mouse back and ear epidermis. The keratin samples were analyzed by isoelectric focusing in the first dimension (IEF; pH gradients 5 to 8) and SDS-polyacrylamide gel electrophoresis in the second dimension (SDS; 8.7% gels). Numbering of spots according to molecular weight corresponds to that of the 1-dimensionally resolved keratin polypeptides in Fig. 2. a to e, keratin polypeptide patterns of back epidermis. a, normal epidermis; b, 1 hr after a single treatment with TPA; c, 8 hr after a single treatment with TPA; d, 14 hr after a single treatment with TPA; e, 48 hr after a single treatment with TPA. Large asterisk in b and c, abnormally focused proteins at the molecular weight level of keratin polypeptides 5 and 6; small asterisk in b and c, prominent acidic proteins at the molecular weight level of actin (A). In c, a 1-dimensional, heavily overloaded run of a keratin sample of back epidermis 8 hr after TPA application is shown. f, the keratin polypeptide pattern of ear epidermis 72 hr after a single treatment with TPA.
Fig. 5. Effect of reversible hyperplasia on the keratin polypeptide pattern of adult mouse back epidermis. Twenty-four mice received a single application of TPA (20 nmol in 100 μl acetone per mouse). A group of 4 acetone-treated mice served as control group. One to 4, 7, and 14 days after the treatment, groups of 4 mice were killed, the control group being sacrificed together with the animals of the 14-day TPA group. Back skins of all experimental animals were depilated, and randomly chosen small skin pieces were processed for routine histology. Epidermis was separated by the acetic acid method, and keratins were extracted from the pooled epidermal fractions and resolved on 8% polyacrylamide gel. Slot A, newborn mouse back epidermis; Slot B, adult mouse back epidermis; Slots C to F, 1 to 4 days after TPA treatment; Slot G, 7 days after TPA treatment; Slot H, 14 days after TPA treatment; Slot I, molecular weight markers bovine serum albumin (BSA) and actin.

Fig. 6. Effect of reversible hyperplasia on the keratin polypeptide pattern of adult mouse ear epidermis. Groups of 4 mice were treated with TPA or acetone as described in legend to Fig. 4, except that the TPA dose was 10 nmol/ear and that ear skin was left without depilation. Slot A, control ear epidermis; Slots B to E, 1 to 4 days after TPA treatment; Slot F, 7 days after TPA treatment; Slot G, 14 days after TPA treatment (7% polyacrylamide gel).
Fig. 7. Effect of permanently sustained hyperplasia on keratin polypeptide patterns of adult mouse back, ear, and tail epidermis. For back epidermis, groups of 12 mice were treated 3 times per week with TPA (10 nmol/mouse), 4-O-methyl-TPA (200 nmol/mouse), or mezerein (5 nmol/mouse). Two weeks after regular treatment, 3 mice of each group were sacrificed either 6 or 24 hr after the last application of the corresponding hyperplasiogen. The remaining animals were sacrificed after 4 weeks of continued treatment. Back skins of all experimental animals were depilated, and randomly chosen small skin pieces were processed for routine histology. Slot A, normal back epidermis; Slot B, 96 hr after a single application of TPA; Slot C, 2 weeks after regular TPA treatment (24 hr after the last application). The keratin patterns after regular treatment with 4-O-methyl-TPA or mezerein were identical to that of Slot C (results not shown). For ear epidermis, groups of 12 mice were treated 3 times per week with TPA (5 nmol), 4-O-methyl-TPA (100 nmol), or mezerein (2.5 nmol) and killed according to the time schedule indicated above. Slot D, normal ear epidermis; Slot E, 96 hr after a single application of TPA; Slot F, 2 weeks after regular TPA treatment (24 hr after the last application); Slot G, 2 weeks after regular mezerein treatment (24 hr after the last application). For tail epidermis, groups of 4 mice were treated 3 times per week with TPA (40 nmol/mouse) or 4-O-methyl-TPA (800 nmol/mouse) for 2 months. A group of 12 animals received daily applications of 200 IU vitamin A acid for 2 weeks. All animals were killed 24 hr after the last application of the corresponding hyperplasiogen. Small skin pieces of randomly chosen tail skin specimen were processed for routine histology. Slot H, normal tail epidermis; Slot I, 2 months after regular TPA treatment; Slot J, 2 months after regular 4-O-methyl-TPA treatment; Slot K, 2 weeks after regular vitamin A treatment. Slot L shows the keratin polypeptide pattern of neonatal mouse tail epidermis, and bovine serum albumin (BSA) and actin were run as molecular weight markers in Slot M (7% polyacrylamide gel).
Fig. 8. In vitro translation and in vivo labeling of keratins. For in vitro translation, polyadenylic acid-containing RNA was purified from newborn mouse epidermis and translated in vitro in a reticulocyte lysate system in the presence of [35S]methionine. To avoid disturbances of the migration of translated keratin polypeptides by system-inherent proteins, 5 translation assays were pooled and extracted twice with a high-salt buffer (1.5 M KCl-10 mM NaCl-2 mM dithioerythritol-0.5% Triton X-100-10 mM Tris-HCl, pH 8.0). Twenty-five µg of newborn mouse keratin were added as carrier to the small, high-salt-insoluble residue and dissolved in 50 µl of a solution containing 5% SDS and 5% β-mercaptoethanol. For in vivo labeling, groups of untreated newborn mice and adult mice, treated once with TPA, received s.c. injections of a 14C-labeled amino acid mixture. Six hr later, the animals were killed, and keratins were extracted from the pooled epidermal tissues. Both the in vitro-translated and in vivo-labeled keratin probes were resolved in a 7% polyacrylamide gel which was prepared for fluorography according to the method of Bonner and Laskey (7). Slots A and B, fluorograms of the translation products from 2 independent series of in vitro translation (Slot A, 0.5 µg polyadenylic acid-containing RNA per assay; Slot B, 1.5 µg polyadenylic acid-containing RNA per assay). Slot C, fluorogram of in vivo-labeled keratin polypeptides of neonatal mouse back epidermis; Slot D, fluorogram of in vivo-labeled keratin polypeptides of adult mouse back epidermis 72 hr after a single treatment with TPA. Slots C and D stem from a separate electrophoretic run, and there are slight differences in the migration distances of the proteins in Slots A to D. Arrowheads, position of keratin polypeptides 2 and 7.
Fig. 9. Two-dimensional analysis of keratin polypeptides translated in vitro or labeled in vivo. The keratin samples analyzed by 1-dimensional electrophoresis in Fig. 8 were investigated by isoelectric focusing in the first dimension (IEF; pH gradients 5 to 8) and SDS-polyacrylamide gel electrophoresis in the second dimension (SDS; 8.7% gel). Gels were prepared for fluorography according to the method of Bonner and Laskey (7). For details of the in vivo labeling procedure and the in vitro translation, see legend to Fig. 8. Numbering of spots according to molecular weights corresponds to that of the 1-dimensionally resolved keratin polypeptides in Fig. 2. a, fluorogram of in vivo-labeled keratin polypeptides of adult mouse back epidermis 72 hr after a single application of TPA; b, fluorogram of in vivo-labeled keratin polypeptides of newborn mouse back epidermis; c, fluorogram of in vitro translation products of polyadenylic acid-containing RNA of newborn mouse epidermis. Asterisk, abnormally focused proteins at the molecular weight level of keratin polypeptides 5 and 6; A, position of actin. d, fluorogram of in vitro translation products after prolonged exposure of the gel.
Changes in Regional Keratin Polypeptide Patterns during Phorbol Estermediated Reversible and Permanently Sustained Hyperplasia of Mouse Epidermis

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