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Keratinocyte Differentiation in Hyperproliferative Epidermis: Topical Application of PPARα Activators Restores Tissue Homeostasis

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We recently showed that topically applied PPARα activators promote epidermal differentiation in intact adult mouse skin. In this study we determined the effect of clofibrate and Wy-14,643, activators of PPARα, on hyperproliferative epidermis in hairless mice, induced either by repeated barrier abrogation (subacute model) or by essential fatty acid deficiency (chronic model). The hyperproliferative epidermis was characterized by an increased number of proliferating cells expressing proliferating cell nuclear antigen. Topical treatment with PPARα activators resulted in a substantial decrease in epidermal hyperplasia in both the subacute and chronic models of hyperproliferation. Following topical treatment, proliferating cell nuclear antigen-expressing cells were restricted to the basal layer, similar to normal epidermis. In hyperproliferative epidermis there was decreased expression of involucrin, profilaggrin-filaggrin, and loricrin as assayed by in situ hybridization and immunohistochemistry. Following topical treatment with PPARα activators staining for these mRNAs and proteins increased towards normal levels. Finally, topically applied clofibrate also increased apoptosis. This study demonstrates that topical PPARα activators have profound effects on epidermal gene expression in hyperproliferative skin disorders. Treatment with PPARα activators normalizes cell proliferation and promotes epidermal differentiation, correcting the cutaneous pathology. This study identifies PPARα activators as potential skin therapeutic agents. Key words: clofibrate/epidermal hyperplasia/involucrin/oricrin/Wy-14,643. J Invest Dermatol 115:361–367, 2000

Regulation of keratinocyte differentiation is still not well understood (Fuchs, 1990; Eckert et al, 1997). Recently, however, it has become clear that ligands of certain nuclear hormone receptors (Mangelsdorf et al, 1995) that form heterodimers with retinoic-X-activated receptor (RXR) are regulators of keratinocyte differentiation. Treatment with ligands, i.e., activators of these receptors (such as vitamin D and retinoic acid), regulate keratinocyte differentiation (Eichner et al, 1996; Fisher and Voorhees, 1996; Kang et al, 1996; Hanley et al, 1997). Furthermore, transgenic mice expressing dominant-negative RXR, or retinoic-acid-activated receptor in the epidermis display defective keratinocyte proliferation and differentiation (Imakado et al, 1995; Saitou et al, 1995; Feng et al, 1997).

Peroxisome-proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily that forms heterodimers with RXR (Isserman and Green, 1990; Schoonjans et al, 1996). The three PPAR isoforms, PPARα, PPARδ, and PPARγ, are expressed in cultured keratinocytes (Rivier et al, 1998). Moreover, PPARα and PPARδ are expressed both in fetal and adult rodent epidermis (Braissant et al, 1996; Braissant and Wahl, 1998). Studies in our laboratory have demonstrated that ligands of PPARα (Schoonjans et al, 1996) stimulate differentiation and inhibit proliferation in cultured human keratinocytes (Hanley et al, 1997). We also showed that PPARα activators accelerate stratum corneum development when added to fetal rat skin explants (Hanley et al, 1997; Kömürös et al, 1998). Moreover, we recently demonstrated that PPARα activators (clofibrate and Wy-14,643) modulate keratinocyte differentiation and gene expression in intact normal skin following topical application (Kömürös et al, 2000). Topical treatment of adult mice with PPARα activators (clofibrate and Wy-14,643) resulted in a decrease in epidermal thickness and increased expression of structural proteins of the upper spinous/granular layers (involucrin, profilaggrin-filaggrin, loricrin). Furthermore, topically applied PPARα activators also increased apoptosis, decreased cell proliferation, and accelerated recovery of barrier function following acute barrier abrogation. PPARα activators did not elicit these epidermal responses in PPARα knockout mice, however (Lee et al, 1995), indicating that PPARα mediates these effects (Kömürös et al, 2000). Furthermore, the epidermis of PPARα−/− mice displayed slightly decreased expression of involucrin, profilaggrin-filaggrin, and loricrin, and also focal parakeratosis, indicative of impaired differentiation (Kömürös et al, 1996).
These observations suggest that PPARα activation is one of the pathways that regulate keratinocyte differentiation in normal epidermis. The aim of this study was to determine whether PPARα activation modulates keratinocyte differentiation in pathologic epidermis, characterized by aberrant cell differentiation and hyperproliferation. We show here that topical applications of PPARα activators, clofibrate and Wy-14,643, result in a normalization of gene expression, together with a normalization of the epidermal morphology, both in subacute and chronic models of hyperproliferative skin. These results suggest that PPARα activators play a role in maintaining tissue homeostasis by modulating cell proliferation, keratinocyte differentiation, and apoptosis in the epidermis.

MATERIALS AND METHODS

Treatments and tissue preparation  We used adult male hairless mice (Simonsen, Gilroy, CA, or Charles River, Wilmington, MA) in this study. Essential fatty acid deficiency (EFAD) mice were produced by feeding an isocaloric essential fatty acid free diet (Dyets, Bethlehem, PA) after weaning for 2 to 3 mo (Man et al., 1993). Subacute epidermal hyperproliferation was induced by repeated barrier abrogation with acetone treatment (Denda et al., 1996). Animals were treated twice a day with acetone until the transepidermal water loss reached 8–10 mg per cm² per h (measured on four or five different spots) as determined with an electrolytic water analyzer (Meeco, Warrington, PA), for 7 d (Denda et al., 1996). To test the effect of PPARα activators on the hyperproliferative skin, EFAD or acetone-treated mice were treated on one side of the flank with 40 μl per cm² of 1 molar clofibrate (Sigma, St. Louis, MO) or with 0.5 molar Wy-14,643 (Sigma), dissolved in propylene glycol:ethanol (7:3 ratio). Animals were treated twice a day, for 3 d. Control animals were treated with vehicle only. Tissue samples were collected for histology, in situ hybridization, or immunohistochemistry as described earlier (Komüves et al., 1998).

Immunohistochemistry  Affinity-purified rabbit antibodies specific for mouse involucrin, profilaggrin-filaggrin, and loricrin were from BabCo (Richmond, CA); affinity-purified biotinylated goat antirabbit IgG was purchased from Vector (Burlingame, CA). The immunohistochemical staining was detected by ABC-peroxidase (Vector) and diaminobenzidine substrate (Vector) as described earlier (Komüves et al., 1998). Omission of the first antibodies or incubation with the substrate solution resulted in no signal, showing the specificity of the detection.

Detection of proliferating cells  The biotinylated anti-proliferating cell nuclear antigen mouse monoclonal antibody was from CalTag Laboratories (Burlingame, CA). The binding of this antibody was detected by Avidin-Alexa (Molecular Probes, Eugene, OR), followed by Sytox Green nuclear counterstain. Sections were analyzed with Leica (Deerfield, IL) TCS laser-scanning confocal microscope.

In situ hybridization  Digoxigenin-labeled RNA probes to detect loricin (Y noncoding region, 200 bases) (Mehrel et al., 1990) and profilaggrin (coding region, 300 bases) (Yuspa et al., 1989) mRNAs were made from linearized cDNA sequences (a gift from S. Yuspa, NIH) using reagents supplied by Boehringer-Mannheim (Indianapolis, IN). In situ hybridization was performed as described earlier (Komüves et al., 1998). Hybridization of DIG-labeled probes to the endogenous mRNA was detected by anti-DIG-alkaline phosphatase (Boehringer-Mannheim), using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium blue substrate. Hybridization with DIG-labeled sense control probes, omission of antisense probes from the hybridization, or incubation with substrate only resulted in no staining.

Detection of apoptosis by TdT-mediated dUTP nick end-labeling (TUNEL) assay  Deparaffinized sections were treated first with 0.5% sodium tetraborohydride for 30 min. TUNEL assay was carried out using the in situ cell death detection kit from Boehringer-Mannheim, following the instructions of the manufacturer. Following the TUNEL reaction the sections were counterstained with Sytox Orange (Molecular Probes, Eugene, OR) nuclear stain. The incorporation of FITC-labeled dUTP was visualized with a Leica (Deerfield, IL) TCS laser-scanning confocal microscope.

Microscopy and imaging  Epidermal thickness was measured on hematoxylin and eosin stained sections using a 20× objective and an ocular micrometer (final magnification 100×). Epidermal thickness was defined as the distance between the basement lamina and the apical surface of the uppermost nucleated keratinocytes, i.e., the border between the stratum granulosum and stratum corneum. Photographs were taken with a Zeiss Axioptot microscope on Kodak (Rochester, NY) Gold 100 print film. Printed images (4 × 5 inches) were digitized with a flatbed scanner, and assembled using Photoshop 3.0 (Adobe Systems, Mountain View, CA) on a Macintosh G3 platform (Apple, Cupertino, CA).

Figure 1. Effects of topical clofibrate treatment on epidermal morphology. Epidermal hyperproliferation was produced by EFAD diet or by repeated barrier disruption in adult hairless mice. The animals were treated twice a day with vehicle or with clofibrate for 3 d. The treatment resulted in a significant decrease in epidermal thickness (see Table 1 for quantitative data). Hematoxylin and eosin staining. Scale bar: 10 μm.
RESULTS

Topical treatment with PPARα activators decrease epidermal thickness in murine models of hyperproliferative skin. To determine the effects of PPARα activators on hyperproliferative skin, we employed two well-characterized murine models of epidermal hyperproliferation (Man et al., 1993; Denda et al., 1996). In the subacute model of epidermal hyperproliferation (Denda et al., 1996), repeated barrier abrogation, twice a day for 7 d, led to an 80% increase in epidermal thickness compared with untreated, control animals (Fig 1, Table I). Similarly, when hairless mice were kept on an essential fatty acid free diet for 2 mo (Man et al., 1993), the epidermal thickness doubled (Fig 1, Table I). The epidermis in both of these models also displayed extensive acanthosis and hyperkeratosis (Fig 1). Following topical treatments with clofibrate (Fig 1) or WY-14,643 (not shown) we noted a substantial decrease in epidermal thickness in both models of epidermal hyperproliferation. No necrotic cells were seen by histologic evaluation of the tissues, however, following these treatments. Whereas in the subacute model a complete recovery was observed (Table I), the epidermis in the EFAD mice remained slightly thicker than the normal control despite a 66% decrease in epidermal thickness (Table I). Epidermal thickness did not change in the untreated side of clofibrate-treated animals and it did not differ from vehicle-treated or untreated hyperproliferative skin (Table I), suggesting that clofibrate exerts its effects locally.

Topical PPARα activators decrease cell proliferation in hyperproliferative skin. We next determined whether the decrease in epidermal hyperplasia could result from decreased cell proliferation. Proliferative cells were identified with an antibody specific for proliferating cell nuclear antigen (PCNA). In normal control hairless mice skin PCNA-positive cells were confined to the basal layer (Fig 2A). In contrast, an expansion of proliferative keratinocytes was seen in both subacute and acute hyperproliferative epidermis (Table II, Figs 2B, E). In both hyperproliferative models topical clofibrate treatment decreased the number of PCNA-positive cells (Table II, Figs 2C, F). Moreover, PCNA-positive cells were found mainly in the basal layer following topical clofibrate treatment (Figs 2C, F). Similar results were seen following topical WY-14,643 treatment (Figs 2D, G). These observations indicate that topically applied PPARα activators downregulate cell proliferation in hyperproliferative epidermis and restore a normal cell proliferation pattern that is restricted to the basal cell layer.

Topical PPARα activator treatment accelerates keratinocyte differentiation in hyperproliferative skin. The altered distribution of proliferative cells in murine hyperproliferative epidermis observed in this study suggested that keratinocyte differentiation could be disturbed. Therefore, we next determined the expression of genes characteristic for terminal keratinocyte differentiation (Fuchs, 1990; Eckert et al., 1997). Involutrin, filagrin, and loricrin mRNA and protein are localized to the upper layers of epidermis in normal hairless mice. This expression and distribution pattern, however, was altered in hyperproliferative epidermis. Whereas involucrin staining was seen in the upper layers (Figs 3A, G), profilaggrin-filaggrin (Figs 3B, H) and loricrin (Figs 3C, I) stained weakly in the hyperproliferative epidermis, especially in EFAD mice. Following topical clofibrate treatment involucrin staining increased somewhat in individual keratinocytes (Figs 3D, J), and the granular nature of the staining was more obvious. Moreover, staining both for profilaggrin-filaggrin (Figs 3E, K) and loricrin (Figs 3F, L) increased in the granular layer following clofibrate treatment, and the staining intensity was similar to that observed in control hairless mice epidermis.

Next we used in situ hybridization to analyze the changes in profilaggrin and loricrin gene expression. Whereas both profilaggrin-
grin mRNA (Figs 4A, E) and loricin mRNA (Figs 4B, F) were present in the upper layers of the stratum granulosum in hyperproliferative epidermis, this staining was weak, particularly for loricin, compared with normal epidermis. Following topical clofibrate treatment a more proximal expression of these genes was observed (Figs 4C, D, G, H). The staining intensity in individual cells was also increased greatly, especially for profilaggrin mRNA (Fig 4I vs 4C, and 4E vs 4G), suggesting increased expression of these genes. These results show that topical clofibrate treatment normalizes the distribution of involucrin, profilaggrin–filaggrin, and loricin proteins, and increases profilaggrin and loricin gene expression in both models of epidermal hyperproliferation.

**Topical PPARα activators increase apoptosis in hyperproliferative epidermis** Apoptosis plays a major role in the elimination of injured or abnormal cells in other tissues as well as in the epidermis (Haake and Polakowska, 1993). We used the TUNEL method, which detects DNA fragmentation, a hallmark of apoptosis, to identify apoptotic cells in the hyperproliferative skin in the models. In the epidermis of untreated normal hairless mice apoptotic cells were occasionally encountered (Fig 5A). Likewise, apoptotic cells were found at the stratum granulosum–stratum corneum interface in the hyperproliferative epidermis of EFAD mice (Fig 5B) or following repeated barrier abrogation (Fig 5D). The number of TUNEL–positive cells increased following topical clofibrate (Figs 5C, F) or Wy-14,643 (Figs 5D, G) treatments, however. Moreover, apoptotic cells not only occurred at the stratum granulosum–stratum corneum interface, but were encountered in the deeper epidermal layers (Figs 5C, D) and in the dermis. These observations indicate a strong increase in apoptosis following topical clofibrate treatment of acute and chronic hyperproliferative skin.

**DISCUSSION**

Several studies have shown that the pattern of keratinocyte differentiation is abnormal in hyperproliferative epidermis in human skin. In psoriasis, a hyperproliferative skin disease, cell proliferation is increased whereas the onset of epidermal differentiation is delayed (Bernerd et al, 1992). The mRNA and protein levels for several genes (such as keratin 15, filaggrin, and loricin), which are normally expressed in terminally differentiating granular keratinocytes, are decreased (Watanabe et al, 1991; Bernerd et al, 1992). Moreover, the gene expression pattern of transglutaminase 1 (Nonomura et al, 1993) and involucrin (Ishida-Yamamoto and Izuka, 1995) is also disturbed, resulting in abnormal cornified envelope formation (Ishida-Yamamoto and Izuka, 1995). Furthermore, in transgenic mice expressing a mutant desmosomal cadherin, which exhibit epidermal hyperplasia, the expression of filaggrin and loricin decreases (Allen et al, 1996). As we have

**Table II. Topical application of a PPARα activator (clofibrate) decreases cell proliferation in hyperproliferative epidermis**

<table>
<thead>
<tr>
<th>Animals</th>
<th>Treatment</th>
<th>PCNA-positive cells per mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Untreated (n = 5)</td>
<td>80 ± 12</td>
</tr>
<tr>
<td>EFAD</td>
<td>Vehicle (n = 4)</td>
<td>164 ± 11</td>
</tr>
<tr>
<td></td>
<td>Clofibrate (n = 6)</td>
<td>77 ± 15³</td>
</tr>
<tr>
<td>Repeated barrier disruption</td>
<td>Vehicle (n = 4)</td>
<td>150 ± 21</td>
</tr>
<tr>
<td></td>
<td>Clofibrate (n = 4)</td>
<td>98 ± 16³</td>
</tr>
</tbody>
</table>

³Data are presented as mean number of PCNA-positive cells ± SEM per mm. The total length of the epidermis analyzed varied between 10 and 16 mm for each sample.

²Significantly different (p < 0.001) as compared to vehicle-treated control.

No significant difference (p > 0.01) as compared to normal untreated control.

**Figure 3. Effects of topical clofibrate treatment on the localization of involucrin, profilaggrin–filaggrin, and loricin proteins in hyperproliferative skin.** Epidermal hyperproliferation was produced by EFAD diet (A–F) or by repeated barrier disruption (G–L) in adult hairless mice. The animals were then treated twice a day with vehicle (A–C, G–I) or with clofibrate (D–F, J–L) for 3 d. Mouse involucrin (A, D, G, J), profilaggrin–filaggrin (B, E, H, K), and loricin (C, F, I, L) were detected using specific antibodies. For details of the immunolocalization see Materials and Methods. Scale bar: 20 μm.
Figure 4. Effects of topical clofibrate treatment on the expression of profilaggrin and loricrin genes detected by in situ hybridization. Epidermal hyperproliferation was produced by EFAD diet (A-D) or by repeated barrier disruption (E-H). The animals were then treated twice a day with vehicle (A, B, E, F) or with clofibrate (C, D, G, H) for 3 d. Profilaggrin (A, C, E, G) and loricrin mRNA (B, D, F, H) was detected with digoxigenin-labeled riboprobes. Scale bar: 20 µm.

described in this study, in two mouse models of pathologic epidermal hyperproliferation, i.e., induced by EFAD or by repeated barrier abrogation, several features of abnormal epidermal keratinocyte differentiation (increased cell proliferation, decreased mRNA levels for profilaggrin and loricrin) also occur. As these features are hallmarks of human hyperproliferative skin diseases such as psoriasis, these murine models may be useful to analyze pathologic mechanisms in hyperproliferative skin disorders. It should be recognized, however, that these murine models do not perfectly mimic psoriasis or other human disorders. In these studies we have elected to use two animal models that are likely to develop epidermal hyperproliferation by different mechanisms. It is hoped that by studying two different pathogenic models the observations reported in this paper will be more likely to be applicable to human disorders.

This study expands the role of PPARα in skin biology as we demonstrate here that epidermal hyperproliferation can be corrected by topical application of clofibrate or WY14,643, PPARα activators (Iseman and Green, 1990; Schoonjans et al., 1996). Topical treatment with PPARα activators not only decreased cell proliferation in hyperproliferative epidermis but increased transformation of the terminally differentiated keratinocytes into corneocytes, as witnessed by the elevated expression of involucrin, filaggrin, and loricrin genes and by an increased incidence of apoptosis. Apoptosis was induced in all layers of the epidermis by topical treatment with PPARα activators. Moreover, the pool of proliferative cells returned to normal, as, following topical PPARα activator treatment, PCNA-positive cells were almost exclusively restricted to basal keratinocytes. These pronounced changes in PCNA immunostaining are probably due to the short half-life of these proteins. Cyclins (recognized by the PCNA antibody) are rapidly degraded as cells cease proliferation (King et al., 1996). Clofibrate treatment also increased the expression of genes required for terminal keratinocyte differentiation (involucrin, profilaggrin, and loricrin). Interestingly, however, the clofibrate-induced changes were more pronounced at mRNA levels than at protein levels. These observations indicate that coordinate regulation of a set of genes (including involucrin, filaggrin, and loricrin) is specifically modulated with PPARα activators. These findings also suggest that the changes induced by
PPARα activators are probably not due to nonspecific, metabolic effects, but are mediated by not yet identified PPARα-response elements. The clofibrate-induced changes in gene expression coupled with the increased apoptosis resulted in a marked decrease in epidermal thickness as a consequence of increased cellular turnover.

Fatty acids, particularly polyunsaturated fatty acids, are ligands of PPARα (Schoonjans et al., 1996). One could therefore postulate that a decrease in essential fatty acids in the EFAD mice results in a reduction of ligands for PPARα, thereby leading to hyperproliferation. This is very unlikely to be the case, however, because our studies of PPARα-deficient mice have not demonstrated a hyperproliferative phenotype (Köműves et al., 2000). Additionally, this deficiency of fatty acids could not explain the effect of PPARα activators in normalizing the epidermis of animals in which the pathology was induced by repeated barrier abrogation.

In an earlier study (Köműves et al., 2000) we found that topical clofibrate treatment decreased cell proliferation and increased apoptosis in normal epidermis. Moreover, PPARα ligands inhibit cell proliferation in human cultured keratinocytes (Hanley et al., 1998) and breast cancer cells (Elstner et al., 1998). Therefore, in general, fatty acids and other PPARα ligands have a cytostatic effect (Pineau et al., 1996; Van den Heuvel, 1999), and induce apoptosis (Chinetti et al., 1998). In extra-epithelial tissues (including the epidermis) PPARα activation regulates tissue homeostasis. In contrast, in rodent liver prolonged treatment with PPARα activators results in hepatic carcinogenesis as a consequence of increased cell proliferation and suppressed apoptosis (Bayly et al., 1994; Gonzalez et al., 1998). These opposing effects of PPARα ligands in hepatic versus nonhepatic tissues suggest the existence of tissue-specific PPARα-response elements, which regulate cell proliferation and apoptosis in a cell- and/or differentiation-specific manner.

The epidermis is a continuously renewing tissue in which homeostasis is maintained by a tightly regulated balance between cell proliferation, cell differentiation, and programmed cell death. Proliferating cells, terminally differentiating cells, and apoptotic cells are spatially and temporally separated in the epidermis. In the normal epidermis proliferating cells are confined to the basal layer, whereas apoptosis occurs at the border of the stratum granulosum and stratum corneum (Haake and Polakowska, 1993; Steiner, 1995). In a plethora of skin diseases (Cerio, 1998), such as localized benign epithelial tumors (keratoderma, epidermal naevi), in papillomas (viral warts, molluscum contagiosum), and in certain secondary inflammatory conditions (psoriasis and lichen planus) normal tissue homeostasis is disturbed, resulting in epidermal hyperproliferation. The data presented here show that PPARα activation is able to counteract epidermal hyperproliferation, suggesting that PPARα activators could be used effectively as therapeutics to treat a variety of skin conditions and skin diseases.

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Figure 5. Effects of topical PPARα activator treatment on apoptosis. Apoptosis was detected in normal control hairless mice (A), or in hyperproliferative skin produced by EFAD diet (B, C, D), or by repeated barrier disruption (E, F, G) in adult hairless mice. The animals were treated twice a day with vehicle (B, E), clofibrate (C, F), or Wy-14,643 (D, G) for 3 d. Apoptotic cells were detected by the TUNEL assay, and the FITC–uridine label was visualized by confocal microscopy. The sections were counterstained with Sytox Orange to detect nuclei (red color). Apoptotic cells are identified by yellow-green nuclei. Scale bars: 50 μm.
PPARα and FXR accelerate the development of the fetal epidermal permeability barrier. *J Clin Invest* 100:705–712, 1997


