Inflammation resolved by retinoid X receptor-mediated inactivation of leukotriene signaling pathways

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ABSTRACT Leukotrienes are implicated in the pathogenesis of diverse, inflammation-driven diseases. Metabolic inactivation of leukotriene signaling is an innate response to resolve inflammation, yet little is known of mechanisms regulating disposition of leukotrienes in peripheral tissues afflicted in common inflammatory diseases. We studied leukotriene hydroxylases (CYP4F gene products) in human skin, a common target of inflammation and adverse drug reactions. Epidermal keratinocytes express at least six CYP4F enzymes; the most highly expressed and highly regulated is CYP4F3A—the main neutrophil leukotriene hydroxylase. Differentiation-specific factors and retinoids are positive CYP4F regulators in vitro, effecting increased leukotriene B4 hydroxylation (inactivation). CYP4F4 expression is up-regulated in situ in hyperproliferative dermatoses—an innate mechanism to repair and restore epidermal barrier competency—and after retinoid therapy. Enhanced CYP4F-mediated inactivation of leukotriene signaling is a previously unrecognized antiinflammatory property of therapeutic retinoids mediated by preferential interactions between retinoid X receptors and CYP4F4 promoter elements in epidermal cells.—Kalsotra, A., Du, L., Wang, Y., Ladd, P. A., Kikuta, Y., Duvic, M., Boyd, A. S., Keeney, D. S., Strobel, H. W. Inflammation resolved by retinoid X receptor-mediated inactivation of leukotriene signaling pathways. FASEB J. 22, 000–000 (2008)

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Leukotrienes are proinflammatory lipids implicated in the pathogenesis of inflammation-driven diseases, including asthma, atopic dermatitis, reperfusion injury, immediate hypersensitivity reactions, adaptive immune responses, rheumatoid arthritis, inflammatory bowel disease, and psoriasis (1, 2). Leukotriene B4 (LTB4) mediates innate immune responses by modulating leukocyte effector functions and production of chemokines and cytokines that amplify inflammation (3). The amplification phase of inflammation is microbiocidal and tissue damaging but normally self-limiting (1). Resolution is effected by innate mechanisms: catabolism of proinflammatory mediators and biosynthesis of proresolving mediators (1, 4). Switching from the tissue-damaging, amplification phase to the tissue-repair and epithelial closure phase is characterized temporally by a switch in production of proinflammatory lipids (e.g., leukotrienes, prostaglandin E2) to proresolving lipids (e.g., lipoxins, resolvins) (1, 4, 5).

Metabolic inactivation of leukotriene signaling has been ignored as a proresolving mechanism due to the dearth of knowledge concerning how catabolic pathways are regulated, especially in peripheral tissues afflicted in common inflammatory diseases. The potent chemoattractant LTB4 is inactivated mainly by hydroxylation at the ω-terminus, catalyzed by cytochromes P450 CYP4F3A and CYP4F2 (CYP gene products) in human neutrophils and liver, respectively (2, 6, 7). Proresolving actions of CYP4F enzymes are further supported by their increased expression in various organ systems following traumatic brain injury or challenge with proinflammatory mediators (6, 8, 9).

Skin is a common target in inflammation-driven diseases (e.g., atopic dermatitis, psoriasis, pemphigus, Goodpasture’s syndrome) and adverse drug reactions (4, 10, 11). Epidermal keratinocytes initiate antigen-independent cutaneous inflammation in response to direct trauma and foreign agents that compromise the epidermal barrier (12). They express pathogen recognition receptors and have important recognized functions in immune surveillance and response, including release of preformed interleukin-1α on injury and production of chemokines and immunoregulatory cytokines that can recruit and activate diverse immune cells (11, 13–15).

Epidermal keratinocytes inactivate LTB4, evidence that their repertoire of expressed CYP genes likely includes CYP4F subfamily members (16–19). The large

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CYP superfAMILY is responsible for oxidative metabolism and disposition of numerous small molecular substrates originating endogenously and exogenously (e.g., vitamins, fatty acids, steroids, environmental toxins, natural products, drugs). The CYP4F subfamily accounts for six of the 57 functional CYP genes in humans and at least seven functional transcripts (CYP4F2, 4F3A, 4F3B, 4F8, 4F11, 4F12, 4F22) (6, 20). In skin, only CYP4F8 and 4F12 have been studied (21–23); neither are logical candidates for LTB4 disposition in vivo (7, 24).

CYP4F substrates include drugs, natural compounds, and a plethora of endogenous ligands, including LTB4, long-chain and very-long-chain fatty acids (25), eicosatetraenoic acids, prostaglandins (6, 7), epoxyeicosatrienoic acids, other fatty acid epoxides and epoxy alcohols (26), tocopherol (27), and phytic acid (28). Besides inactivating leukotriene signaling, CYP4F enzymes likely function in the biosynthesis and catabolism of other immunomodulatory lipids. As CYP4F substrates and products are of considerable medical importance, we investigated factors regulating CYP4F expression and LTB4 inactivation in human skin, a recognized immune organ for which facile in vitro models exist.

**MATERIALS AND METHODS**

**Tissue and cell culture**

Epidermal keratinocytes isolated from discarded, human newborn foreskins were cultured as described (29). Cellular differentiation was initiated when proliferating cultures achieved ~80% confluence (day 0), by adjusting media calcium to 1.4 mM. Differentiating cultures were fed daily with media containing vehicle (0.1% dimethyl sulfoxide) or drugs: 1 nM 23,7,8-tetrachlorodibenzo-p-dioxin (TCDD); 0.1 μM all trans-retinoic acid, unless another concentration is specified; 10 μM dexamethasone; 30 μM β-naphthoflavone; 10 μM rifampicin; 10 μg/ml dapsone; and 0.5 μM monocrotophos. All drugs were from Sigma-Aldrich (St. Louis, MO, USA) except TCDD (AccuStandard Inc., New Haven, CT, USA). Retinoid nuclear receptor ligands LG268 and TTNPB [4-(2,5,6,7-tetrahydro-5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid] were gifts from Peter J. A. Davies (University of Texas-Houston). Biopsy specimens were obtained via Institutional Review Board approved protocols at the University of Texas and Vanderbilt University Dermatology/Dermatopathology. Unaffected and lesional human skin biopsies were taken before and after daily topical application of tazarotene (0.1% gel; Allergan, Irvine, CA, USA); all other biopsies were taken before and after daily topical application of retinoid therapy; other medications utilized Taqman Assays-on-Demand qPCR kits and Universal PCR Master Mix containing AmpErase UNG (Applied Biosystems). These three assays have been described, and validation statistics have been reported for keratinocyte RNA (29). Transcripts encoding retinoid X receptor (RXR) and retinoid A receptor (RAR) isoforms were amplified using Superscript One Step RT-PCR kits (Invitrogen) and transcript-specific oligonucleotides (Supplemental Table 2). β-Actin transcripts were amplified as an internal control. Ethidium bromide visualized products generated after 20, 25, 30, and 35 amplification cycles, resolved on 1.5% agarose gels.

**Gene expression**

Total RNA template was prepared as described (29). Primer Express software (Applied Biosystems, Foster City, CA, USA) aided the design of transcript-specific CYP4F primers and fluorescent probes (Supplemental Table 1). Standard curves were generated with known mass (50 ng to 5 pg) of synthetic, transcript-specific CYP4F amplicons, which enable comparisions of mRNA abundance across CYP4F quantitative real-time PCR (qPCR) assays after normalizing data for 18S rRNA content. IDT DNA Technology synthesized all oligonucleotides. Template (200 ng RNA or known mass of ampiclon) was subjected to reverse transcription in quadruplicate (10 μl reactions containing 1X SSII buffer, 300 nM reverse primer, 500 μM dNTPs, and 10 U Superscript II (Invitrogen, Carlsbad, CA, USA). This cDNA was used neat for all qPCR assays except 18S rRNA (diluted 1:50). Reverse transcriptase was omitted for the negative controls. Reverse-transcription conditions were 50°C for 30 min and 72°C for 5 min. We then added 40 μl of PCR mix (1× PCR buffer, 300 nM forward primer, 300 nM reverse primer, 4 mM MgCl2, 2.5 U Taq polymerase, and 100 nM fluorogenic probe). Cycle threshold (Ct) values for fresh epidermis were <30; those >36 were considered nonspecific.

**Cytookeratin 10, transglutaminase 1, and loricrin assays**

Dewaxed tissue sections were subjected to in situ hybridization. Digoxigenin-labeled cRNA probes (25 ng/ml hybridization buffer) encoding 517–802 bp of CYP4F11 (NM_021187) were prepared using a DIG RNA Labeling Kit, DIG Wash and Block Buffer Set, and digoxigenin antibody (1:500) conjugated to alkaline phosphatase (Roche Applied Science, Indianapolis, IN, USA). Specific hybridization was visualized colorimetrically. Other sections subjected to immunocytochemistry were incubated for 1 h with 5% goat serum in phosphate buffered saline containing 0.25% Triton X-100 and then for 48 h with rabbit antiserum (1:500, 4°C) raised against residues 70–79 of CYP4F2 or residues 260–269 of CYP4F3 (30). Rabbit IgG conjugated to Alexafluor-488 visualized specific binding (Vector Laboratories, Eugene, OR, USA). Other sections were incubated for 30 min with 0.6% hydrogen peroxide and then for 48 h with antiserum specific for neutrophil elastase (EMD Biosciences, San Diego, CA, USA; 1:100, 4°C) or a mixture of both CYP4F antibodies (1:100, 4°C). Vectastain-Elite ABC reagent kit and biotinylated goat anti-rabbit antibody (1:500) were used to visualize specific binding (Vector Laboratories, Burlingame, CA, USA).

**Histology**

Microsomes were prepared from keratinocytes cultures as described (31), with modifications. A Polytron disrupted the cell pellets (PT3100 with PT-DA3007/2 7 mm generator, Brinkmann Instruments, Westbury, NY, USA), which had been scraped in PBS (three 1-min pulses, ~14,000 rpm). Homogenates were transferred to 1.5 ml tubes and centrifuged for 30 min at 10,000 g in a Sorvall Micro-Ultracentrifuge at 4°C (SA100-AT4 rotor; Kendro Laboratory Products, Waltham, MA, USA). The 10,000 g supernatant fractions were centrifuged for 90 min at 100,000 g. The 100,000 g microsomal pellets were stored briefly at −80°C and subjected to LTB4 metabolism assays (100 μg protein/0.1 ml reaction).
Metabolites were resolved by reversed-phase HPLC as described (32). Authentic 20-OH-LTB4 detected at 270 nm was used to estimate the mass of LTB4 metabolites formed. Microsomal proteins (10–40 μg) were also resolved on 4–15% gradient Tris-glycine gels (Bio-Rad, Hercules, CA, USA) and transferred to nitrocellulose membranes, which were blocked overnight and incubated for 3 h with anti-CYP4F2 or anti-CYP4F3A sera (1:500). Chemiluminescence visualized specific complexes formed by antibody-horseradish peroxidase conjugates. Whole cell lysates were subjected to immunoblotting using antiloricrin as described (Covance Research Products, Princeton, NJ, USA; PRB-145P, 1:500) (33).

DNA binding

CYP4F2 promoter elements were identified using TRANSFAC version 4.0 (34). ChiP-IT Chromatin Immunoprecipitation and Enzymatic Shearing kits were used according to the manufacturer’s instructions (Active Motif, Carlsbad, CA, USA). Chromatin was sheared enzymatically (10 min, 37°C) into 200–1000 bp fragments and then immunoprecipitated for 4 h with control mouse IgG, anti-RXR, or anti-RAR IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 2 μg IgG/50 μl sheared chromatin). Immunoprecipitated fractions were amplified using sense (GGGCCAAAGAGTA-GAATTATTTGCCCTCCTGTGACCTC-CYP4F2 probe (5′-3′): GAATTATTTGCCCTCCTGTGACCTC-CYP4F2 probe (5′-3′): GAATTATTTGCCCTCCTGTGACCTC-CYP4F2 probe (5′-3′): GAATTATTTGCCCTCCTGTGACCTC-CYP4F2 probe (5′-3′): GAATTATTTGCCCTCCTGTGACCTC-CTTGGAGTTAAT). Biotinylation efficiency was estimated by dot blot analyses of control oligonucleotides. Protein-DNA complexes were resolved on 5% Tris borate-EDTA gels (Bio-Rad), transferred to nylon membranes, and visualized by chemiluminescence. Some reactions were preincubated for 10 min with 200-fold excess of unlabeled CYP4F2 probe before adding biotinylated CYP4F2. For supershift assays, reactions were preincubated for 10 min with 2 μg RXR-specific or RAR-specific IgG (Santa Cruz Biotechnology) before adding biotinylated CYP4F2.

Statistics

Statistical significance was determined using two-tailed, unpaired Student’s t tests. Probability (P) values <0.05 were considered statistically different.

RESULTS

Differentiation-specific factors regulate epidermal CYP4F expression

We identified transcripts arising from the CYP4F2, 4F3, 4F8, 4F11, and 4F12 genes in human epidermal keratinocytes and studied factors regulating CYP4F expression using a well-characterized in vitro differentiation system (29). In this model, proliferating day 0 cultures represent predominantly a basal cell phenotype. Differentiating day 6 cultures represent predominantly a spinous cell phenotype, evidenced by up-regulated expression of differentiation-specific genes (Supplemental Fig. 1). Cellular differentiation induced mRNA expression levels for CYP4F2 (7-fold), 4F3A (88.5-fold), 4F3B (14-fold), and 4F12 (65.5-fold), compared with levels in proliferating keratinocytes (Fig. 1A). CYP4F3A was the most abundant and most highly regulated transcript (day 6 vs. day 0). Cellular differentiation had no influence on levels of CYP4F11 mRNA; CYP4F8 mRNA was too low to measure.

Fresh epidermal tissue exhibits remarkably similar CYP4F expression profiles (Fig. 1B), demonstrating physiological relevance of the in vitro model. Some differences were expected since epidermis contains four cellular layers corresponding to four differentiation states—basal, spinous, and granular keratinocytes, and anucleated corneocytes. Whereas epidermal cultures are “synchronized” to express one predominant differentiation state. Other factors may influence tissue CYP4F mRNA levels: donor age, site sampled, genetics, and environmental exposures.

CYP4F transcripts and protein immunoreactivity (CYP4F2, CYP4F3A) localize specifically to the cytoplasm of suprabasal keratinocytes in human skin (Fig. 1C). Highest expression localizes to the outer, differentiated cell layers, which is further evidence that differentiation-specific factors regulate epidermal CYP4F expression. We detected little dermal CYP4F expression in fibroblasts, sebocytes, or follicular keratinocytes beneath the level of the sebaceous ducts. Hyperproliferative human skin appeared to express higher CYP4F mRNA and protein levels likely due to an expanded spinous cell layer (increased numbers of CYP4F-expressing keratinocytes vs. normal skin). Another possibility is increased CYP4F protein content per keratinocyte. Concordant results from qPCR, in situ hybridization, and immunofluorescence studies support several conclusions. Human epidermal cells express CYP4F enzymes. The main LTB4 hydroxylase in neutrophils CYP4F3A is also the most highly expressed CYP4F in keratinocytes. The outer, differentiated keratinocytes express the highest CYP4F levels, which are increased further in hyperproliferative (vs. normal) human skin.

Retinoic acid up-regulates epidermal CYP4F expression and LTB4 inactivation

To further study CYP4F transcriptional regulation, keratinocytes were exposed during in vitro differentiation to known hepatic CYP inducers and clinically relevant drug compounds, including nuclear receptor ligands recognized as master regulators of CYP gene expression (35). Transcripts were measured after 6 treatment days, sufficient time for the cultures to form stratified, squa-
mous “epithelia” resembling differentiating spinous cells in vivo (29). Keratinocytes differentiated in the presence of all trans-retinoic acid (0.1 μM) had greater CYP4F2, 4F3A, 4F3B, and 4F11 mRNA levels (Fig. 2A) and greater microsomal CYP4F3A and 4F2 protein content detected by Western blot analyses (vs. vehicle) (Fig. 2D). None of the other tested compounds—2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), dexamethasone, dapsone, 8-naphthoflavone, miconazole, or rifampicin—altered significantly the differentiation-induced expression of CYP4F mRNA (all data not shown). However, early transient changes in mRNA expression would not be detected in these studies. CYP4F12 mRNA levels were unaffected by all treatments, and CYP4F8 was undetectable (data not shown).

Retinoic acid is a negative regulator of epidermal differentiation in vitro (36). In these studies, it blocked the expected increase in cytokeratin 10, transglutaminase 1, and loricrin mRNA and protein at day 6 (Fig. 2D). None of the other tested compounds—2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), dexamethasone, dapsone, β-naphthoflavone, miconazole, or rifampicin—altered significantly the differentiation-induced expression of CYP4F mRNA (all data not shown). However, early transient changes in mRNA expression would not be detected in these studies.

Figure 1. Human epidermis expresses CYP4F mRNA and protein. Cellular mRNA levels (mean±sd) are expressed as the ratio [CYP4F mRNA/18S rRNA]×100. A) CYP4F mRNA levels in epidermal cell cultures (n=6). *P<0.05 (day 6 vs. day 0). N.D., not detectable. B) CYP4F mRNA levels in epidermal tissues (n=4). *P<0.05 (neonatal foreskin vs. adult breast). C) CYP4F mRNA and protein localize preferentially to differentiated epidermal cell layers in situ. Rows 1 and 3 show normal skin; results are representative for neonatal foreskin, adult breast, and adult abdominal skin (n=4 donors). Rows 2 and 4 show hyperproliferative skin (n=2 donors). Blue reaction product visualizes specific hybridization of antisense cRNA sequence common to all CYP4F transcripts (a–d); nuclear-red is the counter stain. Hybridization with sense cRNA is non-specific (q, r). Blue fluorescence visualizes nuclear DNA binding with 4′,6-diamidino-2-phenylindole (e–h). Green fluorescence visualizes specific interactions with anti-CYP4F2 (i–l) or anti-CYP4F3A sera (m–p). Primary antibody was omitted (s, t). Scale bars = 100 μm (a–f, i–j, m–n, q–r); 50 μm (g–h, k–l, o–p, s–t).

In human neutrophils and hepatocytes CYP4F3A and CYP4F2 inactivate LTB4 signaling via catalytic hydroxylation at the LTB4 ω-terminus (7). In differentiating keratinocytes, retinoic acid exposure induced significantly greater rates of NADPH-dependent, microsomal LTB4 hydroxylation (vs. vehicle) (Fig. 2E and Supplemental Fig. 2). This is evidence that retinoid-inducible CYP4F proteins (Fig. 2D) are catalytically active LTB4 hydroxylases.

Retinoid X receptors mediate retinoid-inducible CYP4F expression

Compared with all trans-retinoic acid, 9-cis-retinoic was equally or more efficacious at inducing retinoid-responsive CYP4F transcripts in differentiating keratinocytes. This was observed using submicromolar retinoid concentrations, which are relevant therapeutically (Fig. 3A, B). The precursor retinol was ineffective below 1 μM. Data are shown only for the two most highly regulated transcripts CYP4F2 and 4F3A. These results point to RXR as a critical regulatory factor since 9-cis-retinoic acid is ligand for RXR but not RAR (37). Further supporting this hypothesis, the RXR-specific ligand LG268 (0.1–1.0 μM) induced CYP4F mRNA expression, but the RAR-specific ligand TTNPB at the same concentrations tended to suppress CYP4F expression in differentiating epidermal keratinocytes (Fig. 3C, D).
Results obtained with both natural and synthetic retinoid nuclear receptor ligands point to RXR as a key factor mediating retinoid-inducible CYP4F expression and function in epidermal keratinocytes. Chromatin immunoprecipitation assays show that retinoid nuclear receptor proteins interact specifically with endogenous CYP4F2 promoter elements in differentiating epidermal keratinocytes (Fig. 4A). For the end point, we amplified sequences flanking the putative direct repeat (DR-4) elements in CYP4F2. CYP4F2-specific genomic DNA was amplified preferentially when chromatin from retinoid-acid-treated keratinocytes was immunoprecipitated with RXR-specific antibody (vs. vehicle). In contrast, CYP4F2 genomic DNA was amplified nonpreferentially (regardless of treatment) when the immunoprecipitating antibody was RAR-specific. No products were amplified when the precipitating IgG was nonspecific. These results suggest retinoic acid exposure promotes specific and preferential binding interactions between endogenous RXR and

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**Figure 2.** Retinoic acid induces epidermal CYP4F enzymes and inhibits in vitro differentiation. Human keratinocyte cultures were differentiated for 6 d in the presence of vehicle or drug: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; ATRA, all trans-retinoic acid; Dex, dexamethasone; b-NF, β-naphthoflavone; Micon, miconazole. Cellular mRNA levels (mean±sd; n=3) were normalized for 18S rRNA content and expressed as the ratio [drug/vehicle], where vehicle = 1. A) CYP4F mRNA. B) Cytokeratin 10 (K10), transglutaminase 1 (TGM1), and loricrin mRNA. *P<0.05 (drug vs. vehicle). C) Retinoic acid inhibits loricrin protein expression in differentiating keratinocyte cultures (media Ca²⁺=1.4 mM). Loricrin is undetectable on Western blots when the medium does not support terminal differentiation (Ca²⁺=0.06 mM). D) Retinoic acid induces microsomal CYP4F3A and CYP4F2 content. Antisera interact specifically with keratinocyte protein species (55 kDa) on Western blots. E) Retinoic acid induces NADPH-dependent LTB4 metabolism by keratinocyte microsomes (60 μM LTB4, 20 min, 37°C). Rates are shown for products coeluting with 20-OH LTB4 (mean±sd; n=3 independent determinations from pooled microsomes). Values for vehicle treatment were near the lower assay limit. *P<0.005 (retinoic acid vs. vehicle).

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**Figure 3.** RXR mediates retinoid-inducible epidermal CYP4F expression. Human keratinocyte cultures were differentiated for 6 days in the presence of vehicle or drug: all trans-retinoic acid (ATRA), 9-cis-retinoic acid (9-cis RA), retinol, RAR-specific ligand (TTNPB), or RXR-specific ligand (LG268). CYP4F mRNA are normalized for 18S rRNA and expressed as the ratio [drug/vehicle], where vehicle = 1. Points represent mean values (n=2) for each compound at three concentrations.
DR-4 elements in the proximal CYP4F2 promoter in differentiating epidermal keratinocytes. Binding interactions involving endogenous RAR were not responsive to retinoic acid treatment, although we did not use a quantitative PCR method in this substudy.

Electrophoretic mobility shift assays generated concordant results using cDNA probe encoding CYP4F2 DR-4 promoter elements (Supplemental Fig. 3). Specific protein-DNA complexes were visualized only when nuclear extracts were from retinoic acid exposed keratinocytes. Addition of excess unlabeled probe or RXR-/RAR-specific antibodies disrupted complex formation; these results are consistent with functional RXR-RAR heterodimers in the retinoid treated keratinocytes.

Human epidermis and derived cell cultures express mainly RXR-α and RAR-γ transcripts, regardless of the cultures' differentiation state (Fig. 4B). Other RXR isoforms (β and γ) appeared more abundant in cultured keratinocytes than in epidermal tissue. Other RAR isoforms (α and β) were low or undetectable in both epidermal tissue and cultured cells. Only RXR transcripts appeared to be modulated by retinoic acid in the differentiating (day 6) cultures: RXR-β levels tended to increase, and those for RXR-γ tended to decrease (vs. vehicle). Hence, RXR-α and -β are the isoforms most likely involved in mediating retinoid-inducible CYP4F transcription in human epidermal keratinocytes, via functional heterodimers with RAR-γ or another nuclear receptor.

Enhanced CYP4F-mediated inactivation of LTB4 signaling is an antiinflammatory action of therapeutic retinoids

Results of immunocytochemical analyses (Fig. 5A) suggest retinoid therapy further augments the increased CYP4F expression levels observed in hyperproliferative human skin (Fig. 1C). CYP4F expression tends to be greater in all epidermal layers in retinoid treated (vs. untreated) patients, but the most striking effect of retinoid therapy is demonstrable CYP4F immunoreactivity in basal keratinocytes. This contrasts with negligible CYP4F immunoreactivity in basal keratinocytes in untreated diseased skin.

Up-regulated CYP4F expression in situ and in vitro represents increased capacity to inactivate the potent neutrophil chemoattractant LTB4. A physiological consequence in inflamed skin is to mitigate or reverse the tissue-damaging effects of persistent leukocyte infiltration. In patients with longstanding plaque psoriasis, neutrophils are prominent in the parakeratotic layer forming Munro's microabscess (Fig. 5B). The RAR-β/γ selective retinoid tazarotene effected substantial normalization of epidermal differentiation and loss of neutrophils in psoriatic lesions. These results point toward enhanced CYP4F-mediated inactivation of LTB4 signaling as a previously unrecognized antiinflammatory property of therapeutic retinoids.

DISCUSSION

Retinoids affect numerous signaling pathways in human skin (36). Their antiinflammatory, prodifferentiating, and chemopreventative properties are efficacious
toward diverse disorders including psoriasis, acne, ichthyosis, photoaging, cancer, emphysema, and bronchopulmonary dysplasia in newborns (38–41). We present evidence that retinoids up-regulate a proresolving pathway in human epidermal keratinocytes by mechanisms involving RXR-mediated CYP4F transcriptional activation (Fig. 6). One result is increased epithelial capacity to metabolically inactivate leukotrienes produced by infiltrating neutrophils, thereby antagonizing LTB4 signaling and further neutrophil recruitment. This CYP4F-dependent pathway functions as a physiological stop signal for epithelial inflammation, facilitating the switch to tissue repair and wound healing. Interventions that enhance CYP4F function may augment the therapeutic efficacy of drugs targeting specific etiological factors in inflammation-driven disorders.

Hyperproliferative skin lesions exhibit enhanced CYP4F enzyme levels: CYP4F2 and 4F3A in our studies, CYP4F8 in others (23). This increased capacity to inactivate LTB4 signaling is an innate response to restore tissue homeostasis and epidermal barrier competency. Therapeutic retinoids mimic or augment this innate repair mechanism in diseased skin. CYP4F up-regulation may be a generalized self-defense mechanism effectuated in response to environmentally induced cell damage and chronic inflammatory diseases in genetically predisposed individuals.

This hypothesis is supported by animal studies mod-

Figure 5. In hyperproliferative dermatoses, retinoid therapy is associated with higher CYP4F protein expression in the basal and suprabasal cell layers, loss of neutrophils, and substantial normalization of epidermal differentiation. A) Epidermal CYP4F protein detected in biopsy sections using pooled CYP4F antisera. Panels represent individual patients having the following diagnoses: LDR, lichenoid drug eruption; LDR Rx, patient taking isotretinoin; PR, pityriasis roscacea-like drug reaction; PR Rx, patient taking bexarotene; Ps, psoriasis; Ps Rx, patient taking etretinate. Specificity is demonstrated by omitting antisera (–). Row 1: scale bar = 100 μm. Rows 2–4: scale bar = 100 μm. B) Topical retinoid therapy in patients (n=2) with longstanding plaque psoriasis results in loss of neutrophils in skin lesions, visualized by neutrophil elastase-specific antisera. Patients had biopsies analyzed for unaffected and lesional skin before treatment and then after 14-d treatment with the RAR-selective retinoid tazarotene (Lesional Rx). Arrows indicate elastase-positive neutrophils localized to Munro’s microabscess in lesional skin. Immunoreactivity also localizes to epidermal keratinocytes, which express elastase 1 (64). Scale bars = 100 μm.
elements, activating interactions between RXR and solve inflammation in part by promoting specific chemokine production. Therapeutic retinoids re-genase pathway. LTB4 amplifies inflammation by flamed skin and produce LTB4 expression and function. Neutrophils infiltrate inflammatory actions of retinoid ligands to induce CYP4F synthesis of proresolving lipids (resolution and tissue repair). In this context, CYP4F-expressing keratinocytes implicate RAR-RXR heterodimeric partners must be considered as well (e.g. thyroid hormone nuclear receptors) (54). Several CYP4F genes may be subject to RXR-mediated transcriptional activation, in keratinocytes and in other epithelial cells, since RXR-α has been implicated in retinoid-induced CYP4F2 transcription in hepatic HepG2 cells (53). Other evidences point to RXR as the critical transcription factor. First, retinoic acid exposure modulated RXR but not RAR mRNA levels in keratinocytes. Second, the RXR-selective ligands 9-cis-retinoic acid and LG268 induced greater CYP4F mRNA levels than all trans-retinoic acid. The dominance of RXR-α and RAR-γ isoforms in human epidermis and derived cell cultures is concordant with other human skin studies (36), indicating the in vivo differentiation system is useful and physiologically relevant.

It seems contradictory that retinoic acid up-regulates CYP4F genes, since this epithelial morphogen inhibits sanoid dehydrogenase pathway (52). Mukhtar and colleagues used culture conditions that promote cellular differentiation and induce CYP4F expression and LTB4 hydroxylase activities. In fact, the majority of epidermal CYP4F transcripts are up-regulated by differentiation-specific factors, corroborating the recent localization of CYP4F8 and CYP4F12 to the outer, differentiated layers of human skin (21, 22).

Retinoid-inducible CYP4F2 expression was first studied in hepatic and myeloid cells (30, 53). Recently, CYP4F8 was detected at higher levels in retinoic acid-treated human skin (23). We show retinoid ligands up-regulate several CYP4F transcripts in keratinocytes, and we present the first mechanistic evidence that pharmacological retinoic acid exposure promotes preferentially binding interactions between RXR (presumably α-β isoforms) and the proximal CYP4F2 promoter in human epidermal cells. Gel shift assays also implicate RAR binding interactions, presumably RAR-RXR heterodimers. If RXR heterodimers mediate retinoid-inducible CYP4F2 transcription in vivo, other heterodimeric partners must be considered as well (e.g., thyroid hormone nuclear receptors) (54). Several CYP4F genes may be subject to RXR-mediated transcriptional activation, in keratinocytes and in other epithelial cells, since RXR-α has been implicated in retinoid-induced CYP4F2 transcription in hepatic HepG2 cells (53). Other evidences point to RXR as the critical transcription factor. First, retinoic acid exposure modulated RXR but not RAR mRNA levels in keratinocytes. Second, the RXR-selective ligands 9-cis-retinoic acid and LG268 induced greater CYP4F mRNA levels than all trans-retinoic acid. The dominance of RXR-α and RAR-γ isoforms in human epidermis and derived cell cultures is concordant with other human skin studies (36), indicating the in vivo differentiation system is useful and physiologically relevant.

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![ Diagram depicting the novel antiinflammatory actions of retinoid ligands to induce CYP4F expression and function. Neutrophils infiltrate inflamed skin and produce LTB4 via the 5-lipoxygenase pathway. LTB4 amplifies inflammation by recruiting leukocytes and stimulating cytokine and chemokine production. Therapeutic retinoids re-genase pathway. LTB4 amplifies inflammation by flamed skin and produce LTB4 expression and function. Neutrophils infiltrate inflammatory actions of retinoid ligands to induce CYP4F synthesis of proresolving lipids (resolution and tissue repair). In this context, CYP4F-expressing keratinocytes implicate RAR-RXR heterodimeric partners must be considered as well (e.g. thyroid hormone nuclear receptors) (54). Several CYP4F genes may be subject to RXR-mediated transcriptional activation, in keratinocytes and in other epithelial cells, since RXR-α has been implicated in retinoid-induced CYP4F2 transcription in hepatic HepG2 cells (53). Other evidences point to RXR as the critical transcription factor. First, retinoic acid exposure modulated RXR but not RAR mRNA levels in keratinocytes. Second, the RXR-selective ligands 9-cis-retinoic acid and LG268 induced greater CYP4F mRNA levels than all trans-retinoic acid. The dominance of RXR-α and RAR-γ isoforms in human epidermis and derived cell cultures is concordant with other human skin studies (36), indicating the in vivo differentiation system is useful and physiologically relevant.

It seems contradictory that retinoic acid up-regulates CYP4F genes, since this epithelial morphogen inhibits
other CYPs also expressed preferentially in differentiating keratinocytes (e.g., CYP2C18, 2C19, 2C9, 2W1, 3A4, 4B1) (17). Presently, no information is available on whether endogenous retinoid ligands regulate CYP4F genes. Inferences from pharmacological studies are tenuous because 9-cis-retinoic acid isomerizes to all-trans-retinoic acid (36) and because retinoid exposure induces retinoid-metabolizing CYPs and other enzymes that modulate the cellular levels and molecular entities of biologically active retinoids (37, 55, 56). The physiological relevance of 9-cis-retinoic acid remains controversial, and other eicosanoid or fatty acid ligands might activate RXR in vivo (37).

Mutations in two human genes provide compelling evidence that normal epidermal barrier function requires CYP4F catalysis to inactivate eicosanoids arising from the 5-lipoxygenase/LTB4 pathway and the 12(R)-lipooxygenase/hepoxilin pathway (57, 58). First, mutations in ALDH3A2 in Sjogren-Larsson syndrome are responsible for a spectrum of mild to serious neural and cutaneous defects (10). The fatty aldehyde dehydrogenase encoded by ALDH3A2 converts the LT4B4 aldehyde intermediate to 20-COOH LT4B4, a catalytic step also attributed to CYP4F leukotriene hydroxylases. Phenotypes of Sjogren-Larsson patients demonstrate the importance of efficient leukotriene inactivation. Second, type 3 lamellar ichthyosis patients have mutations in CYP4F2 (59). Since CYP4F2 and CYP4F3B can hydroxylate long-chain and very-long chain fatty acids (25), these genetic studies raise the question whether one or more CYP4F enzymes are required to catalyze ω-hydroxylation of very long chain fatty acids that are critical structural components of the cornified cell envelope in keratinizing epithelia.

Recent studies also implicate CYP4F enzymes in biosynthesis of the novel proresolving lipid resolin ReV1 (5S,12R,18Rtrihydroxy eicosapentaenoic acid). The proposed biosynthetic pathway generating ReV1 from eicosapentaenoic acid (C20:5n-3) involves an unidentified cytochrome P450 (1, 4, 5, 60–62). In vitro studies now establish that CYP4F8 and 4F12 catalyze ω3-hydroxylation and (or) epoxidation of long-chain fatty acids (C20:4n-6, 22:5n-3, and 22:6n-3) (24). These results are important because they imply CYP4F enzymes may be responsible for generating ReV1 intermediates in humans (18Rhydroxy and 5,6-epoxy-18R-hydroxy eicosapentaenoic acid).

In addition to being the most common target affected by inflammation (4), skin provides physiologically relevant and facile models for further studies to elucidate pathways regulating the synthesis and disposition of immunomodulating eicosanoids. Genetic studies now show linkage between CYP4F2 and CYP4F3A gene variants and celiac disease, a chronic inflammatory disorder of the gastrointestinal tract (63). We surmise that induction of CYP4F enzymes may be a general mechanism of how diverse epithelial cells influence the course of inflammatory disease and recovery from environmentally induced cell damage.

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