Differentiation-Specific Factors Modulate Epidermal CYP1–4 Gene Expression in Human Skin in Response to Retinoic Acid and Classic Aryl Hydrocarbon Receptor Ligands

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ABSTRACT

Human epidermal keratinocytes express subsets of cytochromes P450 (P450) (CYP gene products) that are strongly up-regulated, not regulated, or down-regulated by differentiation-specific factors. We investigated how drug exposure affects epidermal expression of CYP1–4 genes, which encode many drug-metabolizing P450s. Real-time polymerase chain reaction (PCR) assays measured CYP1–4 mRNA levels in epidermal keratinocytes differentiated in vitro in the presence of drug or vehicle for 6 days. We confirmed the spinous phenotype at day 6 by changes in cellular morphology and up-regulation of cytokeratin 10 and transglutaminase (TGM)1 mRNA in the differentiating keratinocytes. Effects of drug exposure depended on the influence of differentiation-specific factors in controlling epidermal CYP1–4 expression. CYP2C18, 2C19, 2C9, 2W1, 3A4, and 4B1 are up-regulated by cellular differentiation; mRNA levels for these CYP genes were inhibited in differentiating keratinocytes exposed to retinoic acid and aryl hydrocarbon receptor (AhR) ligands. These same drugs affected ≤2-fold change or even augmented mRNA levels for CYP genes that are not regulated by differentiation (CYP2U1, which is expressed at highest levels in undifferentiated keratinocytes. The clinically relevant drugs miconazole, dexamethasone, rifampicin, and dapsone had little effect on CYP1–4 mRNA levels under assay conditions. The AhR ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin also up-regulated keratinocyte TGM1 mRNA in a concentration- and time-dependent manner. This effect was blocked by the AhR antagonist resveratrol. These findings implicate AhR-dependent up-regulation of TGM1 mRNA in differentiating keratinocytes as one mechanism contributing toward chloracne in humans exposed to toxic levels of dioxin.

Structural and biochemical features of skin are specialized toward its major protective functions as a barrier to the host environment. In addition to regulating water loss and body temperature, skin has mechanisms for self-renewal and for surveillance and repair of cell damage due to biological, physical, and chemical exposures. Epidermal keratinocytes constitute the major reservoir of cutaneous cytochromes P450 enzymes (CYP gene products) involved in oxidative metabolism and disposition of endogenous and foreign compounds, including environmental toxins, natural products, and drugs. Most drug-metabolizing cytochromes P450 are members of the CYP1–4 gene families, which number 35 (61%) of the 57 putatively functional CYP genes in humans (Nelson et al., 2004). Major detoxifying organs such as the liver rank among tissues containing the highest concentrations of cytochromes P450; much lower concentrations are found in peripheral tissues such as skin. Liver and skin express many of the same P450s, but an important difference is the most abundant hepatic P450s tend to be expressed at relatively low levels in cutaneous tissues and vice versa (Du et al., 2006). This tissue-specificity suggests unique adaptation to different environmental exposures and different P450 protein

ABBREVIATIONS: P450, cytochromes P450; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; AhR, aryl hydrocarbon receptor; aTRA, all-trans-retinoic acid; β-NF, β-naphthoflavone; qPCR, quantitative real-time polymerase chain reaction; TGM, transglutaminase; HRP, horseradish peroxidase; AP1, activator protein 1.
functions depending on specialized (differentiated) cellular functions.

Therapeutic compounds used in dermatological practice include many substrates, inducers, and inhibitors of P450 proteins, including antibiotics, antifungal, and other antifungal agents; vitamins; steroids; coal tar extracts; psoralsens; and ultraviolet light (Ahmad and Mukhtar, 2004). Drug substrates often induce P450 enzymes responsible for their metabolism (Levin et al., 1972). There is little quantitative information how drug exposure affects P450 expression in human skin. This cannot be predicted from hepatic cell studies because of quantitative and qualitative differences in P450 expression in these organ systems. Due to the heterogeneous nature of stratified epidermis, cells in different epithelial layers (i.e., different differentiation states) are adapted for different specialized cellular functions and thus express different sets of genes. Hence, cells in different epithelial layers can respond differently to environmental agents. An example is the well studied induction of polycyclic aromatic hydrocarbon hydroxylases (CYP1 family enzymes) by benzo(a)pyrene and other polycyclic aromatic hydrocarbons in human and rodent skin (Levin et al., 1972; Ahmad and Mukhtar, 2004). Differentiating keratinocytes are more responsive than proliferating keratinocytes to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a potent toxin and classic aryl hydrocarbon receptor (AhR) ligand. The AhR target gene CYP1A1 is strongly and preferentially up-regulated in differentiating keratinocytes in response to TCDD exposure (Swanson, 2004).

In previous studies, we showed that human epidermal cell cultures express 13 (of a panel of 15) CYP1–4 genes. By manipulating and monitoring the differentiation state of these cultures, we showed that nearly one-half of these were expressed preferentially in differentiating cells (Du et al., 2006). Expression levels of the other P450s varied little (±2-fold) regardless of differentiation state. Using this characterized model of keratinocyte differentiation, we investigated effects on CYP1–4 gene expression when differentiating epidermal cells are exposed to drug compounds relevant to clinical dermatology practice or foreign compounds that alter normal epidermal differentiation. We report that the nature of the drug-induced regulation depends on the influence of differentiation-specific factors.

### Materials and Methods

#### Cell Cultures.
Keratinocytes isolated from discarded, human newborn foreskins were expanded to passage 3 in Complete medium 154-CF (Cascade, Portland, OR; supplemented with kit S-0001-K), adjusted to 0.05 mM Ca<sup>2+</sup> as described previously (Du et al., 2006). At ~75% confluence, proliferating cultures were either passaged or media calcium was adjusted to 1.4 mM to initiate cellular differentiation (day 0). Differentiating cultures were fed daily with media containing vehicle (0.1% dimethyl sulfoxide) or the following drugs: 1 or 10 nM TCDD, 100 nM all-trans-retinoic acid (atRA), 10 μM dexamethasone, 30 μM β-naphthoflavone (β-NF), 10 μM rifampicin, 10 μg/ml dapsone, 0.5 μM miconazole, 1 μM β-naphthoflavone, and 10 μM resveratrol. All drugs were from Sigma-Aldrich (St. Louis, MO), except TCDD (AccuStandard Inc., New Haven, CT). Keratinocyte cultures were prepared for paraffin embedment as described previously (Du et al., 2006). Tissue sections (5 μm in thickness) were sampled at 300-μm intervals (5–10 blocks sampled per treatment). Hematoxylin and eosin stain was used to evaluate cellular morphology.

#### Quantitative, Real-Time-Polymerase Chain Reaction.
Total RNA was purified using NucleoSpin RNA II kits (BD Biosciences, San Jose, CA), subjected to a second DNase digestion step (DNA-free kit; Ambion, Austin, TX), and reverse-transcribed using a High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Real-time qPCR assays were performed using Taqman Assays-on-Demand and Universal PCR Master Mix containing AmpliFlair UNG (Applied Biosystems), according to the manufacturer’s recommended amplification conditions. We reported validation statistics for all of the P450 assays used in these studies, and the cytokeratin 10, transglutaminase (TGM1), and loriacin assays (Du et al., 2006).

In a separate substudy, we measured TGM1 mRNA levels and two additional assays not previously validated for keratinocyte RNA in our laboratory (Applied Biosystems assay identification number Hs00162752_ml1 for TGM3 and Hs00185862_ml1 for TGM5). To compensate for lower TGM3 and TGM5 expression levels (versus TGM1), the reverse transcription reactions contained 5 μg of RNA/100 μl. Amplification efficiency values [E = 10(−1/slope)] for this substudy were 1.95 (97.5%) for TGM1, 2.01 (100.5%) for TGM3, 2.14 (106.8%) for TGM5, and 2.03 (101.6%) for 18S rRNA.

Data were collected and analyzed using a Prism 7900HT Sequence Detection System and SDS 2.1 software (Applied Biosystems), and then data were normalized for 18S rRNA levels as described previously (Du et al., 2006). One-tailed t tests were used to evaluate treatment differences. One-sample t tests were used to analyze treatment differences when P450 mRNA levels are expressed as the ratio [drug - vehicle]. In this case, vehicle control values were set to 1.0.

#### Western Blot Analyses.
Western blots were prepared as described previously (Ladd et al., 2003). Nitrocellulose membranes containing 30 μg of protein/lane were 1) blocked overnight at 4°C with milk buffer (5% fat-free powdered milk in phosphate buffer saline containing 0.1% Tween 20); 2) incubated 5 h in milk buffer containing AhR antibody diluted 1:1000 (MAI-514; ABX-Afinity BioReagent, Golden, CO); and 3) incubated 1 h in milk buffer containing horseradish peroxidase (HRP)-conjugated anti-mouse-IgG diluted 1:10,000 (A2554; Sigma-Aldrich). Chemiluminescence visualized the immunoreactive proteins on Kodak X-OMAT AR film (Eastman Kodak, New Haven, CT). Membranes were stripped and rebotted using CYP1A1 antibody diluted 1:500 (H-70, sc20772; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and HRP-conjugated anti-rabbit-IgG diluted 1:10,000 (A0545; Sigma-Aldrich). Human TGM1 antibody was used at 1:500 (BT-621; Biomedical Technologies, Stoughton, MA) with signal amplification (SuperPicture Polymer Detection diluted 1:10; Zymed Laboratories, San Francisco, CA). Keratinocyte nuclear extracts were prepared using NucBuster (71193-3; Novagen, Madison, WI). Blots containing nuclear proteins were probed with antibodies to activator protein (AP1) proteins (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), subjected to a second DNase digestion step (DNA-free kit; Ambion, Austin, TX), and reverse-transcribed using a High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Keratinocyte nuclear extracts were prepared using NucBuster (71193-3; Novagen, Madison, WI). Blots containing nuclear proteins were probed with antibodies to activator protein 1 (AP1) proteins (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:1000 as described previously (Mehic et al., 2005); c-Fos (D-1), sc-8047; Fos B (C-11), sc-8013; Fra-1 (N-17), sc-183; Fra-2 (L-15), sc-171; and c-Jun (N), sc-45; Jun B (C-11), sc-8051; and Jun D (D-292), sc-74.

#### TGM-Mediated Cross-Linking.
Cross-linking activities were measured using the in situ TGM enzyme-linked immunosorbent assay as described and validated previously (Ladd et al., 2003), with the following modifications. The detection system used mouse antibiotin diluted 1:4600 (200-002-096; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and SuperPicture Polymer Detection diluted 1:20 (Zymed Laboratories) for signal amplification. To ensure linearity of response, assay plates were coated with differing amounts (0.01–1.0) of whole cell lysates for each unknown sample. Data are expressed as absorbance units (A<sub>450 nm</sub>) per microgram of protein.

### Results

#### Regulation of CYP1–4 Genes Expressed Preferentially in Differentiating Epidermal Keratinocytes.
Differential-specific factors strongly regulate expression of
the CYP2C18, 2C19, 2C9, 2W1, 3A4, and 4B1 genes in human epidermal keratinocytes (Du et al., 2006). In this study, we investigated how epidermal CYP1–4 expression is altered by exposure to known P450 inducers and clinically relevant drug compounds, during the process of cellular differentiation. Cells were differentiated for 6 days in the continuous presence of vehicle or drugs, including retinoic acid, β-naphthoflavone, TCDD, miconazole, dexamethasone, rifampicin, and dapsone. This exposure time was selected because, after inducing differentiation, nearly 1 week is required for the epidermal cultures to form a stratified “epithelium” resembling spinous cells in vivo (Du et al., 2006). In the day 6 cultures, P450 mRNA levels increased on average 113-fold for CYP2C18, 27-fold for CYP2W1, and 356-fold for CYP4B1 (day 6 vehicle versus day 0). CYP2C19, 2C9, and 3A4 mRNA levels also increased markedly, compared with the very low or nondetectable values measured in proliferating keratinocytes at day 0 (data not shown).

Figure 1, A and B, shows effects of drug treatments on mRNA levels for this subset of CYP genes, measured at day 6 of in vitro differentiation. Retinoic acid is a negative regulator of epidermal differentiation in vitro (Fisher and Voo- rhees, 1996). In this study, retinoic acid strongly inhibited the differentiation-induced up-regulation of CYP2C18, 2W1, and 4B1 mRNA in human epidermal keratinocytes (versus vehicle; Fig. 1, A and B). Already near the lower assay limits in vehicle-treated cultures, CYP2C19, 2C9, and 3A4 mRNA levels were reduced further or were undetectable in retinoic acid-treated cultures (data not shown).

The P450 inducer β-naphthoflavone also inhibited the differentiation-induced up-regulation of CYP2C18, 2C19, 2C9, 2W1, 3A4, and 4B1 in human epidermal keratinocytes (versus vehicle; Fig. 1, A and B) (all data not shown). The toxin TCDD had similar effects, with one exception. Instead of being suppressed, CYP2W1 mRNA levels tended to be greater (≈2-fold) in TCDD-treated keratinocytes. None of the other drug treatments—miconazole, dexamethasone, rifampicin, and dapsone—interfered substantially with the differentiation-induced expression of these six CYP1–4 genes (all data not shown). These results strongly suggest that differentiation-specific factors are major determinants governing epidermal expression of the CYP2C18, 2C19, 2C9, 2W1, 3A4, and 4B1 genes in human skin.

Regulation of CYP1–4 Genes Expressed at Similar Levels in Proliferating and Differentiating Epidermal Keratinocytes. The differentiated keratinocyte phenotype

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has modest (≤2-fold change) or no influence on mRNA expression levels of seven CYP1–2 genes, including CYP2J2, 2S1, 1A1, 1A2, 1B1, 2D6, and 2E1 (Du et al., 2006). Typical for this CYP gene subset, CYP1B1 mRNA levels increased 1.8-fold during in vitro differentiation in this study; CYP2S1 and 1A1 mRNA levels decreased by one-half (day 6 vehicle versus day 0). CYP2U1 was identified previously as the only CYP gene, of 15 CYP1–4 genes studied, that was expressed preferentially in proliferating epidermal cells (Du et al., 2006). In this study, CYP2U1 mRNA levels decreased on average by one-fifth during in vitro differentiation (data not shown).

Figure 1, C to F, shows effects of drug treatments on cellular mRNA levels for this subset of eight CYP genes, measured at day 6. Retinoic acid induced CYP2S1 (4-fold), CYP2U1 (1.4-fold), and CYP1B1 (8-fold) mRNA levels (versus vehicle). This is a different and opposite effect compared with the previous P450 subset. Retinoic acid treatment also increased CYP1A2 mRNA levels, which were undetectable in vehicle-treated cultures. CYP2E1 and 2D6 mRNA levels were unaffected, whereas CYP2J2 mRNA levels were lower by one-half, in retinoic acid treated cultures (versus vehicle) (all data not shown).

Rifampicin tended to up-regulate CYP1 mRNA expression 2.6- to 3-fold (p = 0.07 versus day 6 vehicle), but it had little effect on the other P450s in this group (data not shown). Miconazole, dexamethasone, and dapsone had no significant effects on epidermal expression of CYP2J2, 2S1, 1A1, 1A2, 1B1, 2D6, 2E1, or 2U1 (all data not shown).

The classic AhR ligands β-naphthoflavone and TCDD induced profoundly expression of all three CYP genes, including CYP1A2, which was undetectable in vehicle-treated cultures (Fig. 1, E and F). The -fold change in CYP1 mRNA levels far surpassed that for all other drugs. We measured 724-, 3327-, and 3104-fold increases in CYP1A1 mRNA levels in three individual, TCDD-treated cell pools. Expression levels of other P450s in this group were largely unaffected by exposing differentiating epidermal cells to AhR ligands (not all data shown).

Only β-naphthoflavone and TCDD specifically activated keratinocyte AhR, leading to induction of epidermal CYP1A1 mRNA and protein. CYP1A1 protein expression was not detected in proliferating (day 0) epidermal cells or in vehicle-treated differentiating cells measured at day 6 by Western blot analyses (Fig. 2). CYP1A1 was induced profoundly by exposure to both AhR ligands, corroborating changes in epidermal CYP1 mRNA levels. The concomitant down-regulation of AhR protein is also evidence of AhR activation (Fig. 2).

**TCDD Specifically Up-Regulates TGM1 mRNA and Protein Expression and TGM Cross-Linking Activities in Differentiating Keratinocytes.** The ability of AhR ligands to induce epidermal CYP1A1 expression depends on cellular differentiation state; differentiating keratinocytes cells have greater capacity to respond to TCDD. In turn, TCDD exposure alters the normal differentiation program of epidermal cells (Jones and Reiners, 1997; Loertscher et al., 2001; Swanson, 2004). Considering these interactions, we monitored effects of drug exposure on the differentiation state of the cultures, assessed by changes in cellular morphology (Fig. 3) and up-regulated expression of differentiation-specific gene markers (Fig. 4).

Differentiating keratinocyte cultures were stratified at day 6 (vehicle; Fig. 3A). The number of cell nuclei counted vertically was greater at attachment sites of the original colonies, compared with areas between expanding colonies. Cells in the outer layers were flattened, and granules were sometimes visible. These structural features are consistent with a spinous phenotype, as are the induced mRNA expression levels for TGM1 (11-fold), cytokeratin 10 (732-fold), and loricrin (164-fold) (day 6 versus day 0; data not shown). TGM1 is the major TGM isoform in keratinizing epithelia and a marker of differentiating keratinocytes (Lorand and Graham, 2003). Cytokeratin 10 and loricrin mRNA are specific markers of the spinous and granular cell layers of the epidermis, respectively.

Retinoic acid treatment, the negative control in these experiments, effectively blocked epidermal in vitro differentiation. Although retinoic acid-treated cultures were stratified at day 6, the epithelium seemed more compact, and nuclear chromatin seemed more condensed (Fig. 3B). Retinoic acid blocked the differentiation-induced up-regulation of all three differentiation-specific gene markers as well as TGM-mediated cross-linking activities in epidermal keratinocytes (versus vehicle) (Fig. 4, A–D).

Both AhR ligands altered the differentiated cell phenotype at day 6, effecting more extreme cell flattening, nuclear chromatin condensation, hyperkeratinization, and parakeratosis (Fig. 3C; β-naphthoflavone data not shown). These epithelial sheets were exceptionally fragile, and they nearly always fractured during sample processing. The thin, stratum cor-
neum-like layer separated cleanly from the rest of the epithelium, or the fracture occurred one cell layer lower. Abnormal differentiation was further evidenced by the ability of \( \alpha \)-naphthoflavone to suppress up-regulation of cytokeratin 10 and loricrin mRNA (versus vehicle) (Fig. 4, A and B). Whereas TCDD exposure also blocked up-regulation of cytokeratin 10, it had the singular and unexpected effect to induce specifically TGM1 mRNA levels (versus vehicle) (Fig. 4C). This was functionally significant since TCDD treatment (but not \( \alpha \)-naphthoflavone or other drugs) increased (9-fold) TGM-mediated cross-linking in situ (Fig. 4D). Western blot analyses showed that vehicle-treated keratinocytes exhibit time-dependent increases in TGM1 protein (90 kDa) during in vitro differentiation, and TCDD exposure further augments TGM1 protein expression levels in a concentration-dependent manner (Fig. 4E).

Miconazole, dexamethasone, rifampicin, and dapsone had no measurable effects in these studies at the concentrations tested (all data not shown). However, the outer cell layer of miconazole-treated cultures seemed slightly hyperkeratinized (Fig. 3D).

TCDD-Induced Up-Regulation of the Differentiation-Associated TGM Isoforms in Epidermal Keratinocytes. It is unclear how TCDD regulates TGM1 mRNA expression since regions of the TGM1 promoter studied thus far lack the xenobiotic response element core sequence (5′-CACCC-3′). In contrast, TGM enzyme activities levels are governed by multiple mechanisms, including regulation at transcriptional and post-translational levels. Differentiating keratinocytes express at least three TGM isoforms (TGM1, TGM3, and TGM5) that cross-link cornified cell envelope precursor proteins (Lorand and Graham, 2003). We investigated whether mRNA expression of other TGM isoforms was also regulated by TCDD and whether AhR was involved.

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**Fig. 3.** Drug-induced changes in cellular morphology of adherent human epidermal cell cultures. Epidermal keratinocytes were differentiated in vitro for 6 days in the presence of 0.1% dimethyl sulfoxide (Veh) (A), 0.1 \( \mu \)M atRA (B), 1 nM TCDD (C), and 0.5 \( \mu \)M miconazole (MICON) (D). All of the images were captured using a 40× oil immersion objective. Scale bar (D), 50 \( \mu \)m.

**Fig. 4.** The AhR ligand TCDD, but not \( \alpha \)-naphthoflavone, specifically up-regulates TGM1 mRNA and protein and TGM-mediated cross-linking in differentiating epidermal cells. Human epidermal keratinocytes were differentiated in vitro in the presence of drug or vehicle, as described in Fig. 1 legend. qPCR measured mRNA levels at day 6; an enzyme-linked immunosorbent assay measured in situ TGM cross-linking activities at day 8, using the TGM substrate biotinylated cadaverine (1 mM). A, cytokeratin 10 mRNA (K10) levels, a spinous cell marker. B, loricrin mRNA levels, a granular cell marker. C, TGM1 mRNA levels, a differentiating keratinocyte marker. D, in situ TGM-mediated cross-linking activities. E, Western blot showing TCDD-induced TGM1 protein levels. Day 0 control lane represents proliferating epidermal cells. Arrowhead indicates 88-kDa molecular size marker. Each bar represents the mean ± S.D. (n = 3). Significance levels (p values) are for drug versus vehicle: a, \( p \leq 0.005 \); b, \( p \leq 0.05 \); and c, \( p \leq 0.10 \).
In vehicle-treated cells, TGM1 mRNA is most abundant and achieves maximal levels during the first week of in vitro differentiation when the cultures have predominantly a spinous phenotype (Fig. 5A). Differentiation also up-regulates TGM3 and TGM5 mRNA (Fig. 5, B and C), but maximal levels are not achieved until at least the second week of in vitro differentiation (granular phenotype; not all data are shown). TGM7 mRNA levels were too low to measure in the differentiating cultures, and TGM2 was excluded because it is expressed preferentially in proliferating cells (data not shown).

Exposure to TCDD effected concentration- and time-dependent changes in TGM1 mRNA in differentiating keratinocytes (versus vehicle; Fig. 5A). Greater induction of TGM1 mRNA was measured at 10 nM TCDD (8-fold versus vehicle), and maximal responses occurred earlier (day 4). Slower, sustained induction of TGM1 mRNA was measured at 1 nM TCDD (4-fold versus vehicle), with maximal responses at day 6. The different kinetics for different TCDD concentrations may reflect differences in the rates of down-regulation or protein turnover, with respect to AhR or other regulatory proteins mediating TCDD effects (Harper et al., 2006).

Differentiating epidermal cells expressed lower levels of TGM3 and TGM5 mRNA, suggesting that in situ cross-linking measured in these studies was due mainly to TGM1. Exposure to TCDD induced concentration- and time-dependent changes in TGM3 and TGM5 mRNA expression (Fig. 5, B and C). At 10 nM TCDD, maximal induction (5-fold versus vehicle) occurred by day 4 for both TGM3 and TGM5 mRNA levels. At 1 nM TCDD, maximal increases (10-fold) in TGM3 mRNA levels were measured at day 2, and maximal increases (3-fold) in TGM5 mRNA levels were measured at day 4 (versus vehicle).

Next, we treated keratinocytes with TCDD alone and in combination with a competitive AhR antagonist (10 μM resveratrol or 1 μM α-naphthoflavone). Resveratrol was more efficacious at the drug concentrations tested; at 10 μM, α-naphthoflavone can act as a weak AhR agonist (Santostefano et al., 1993). Resveratrol effectively blocked TCDD-induced CYP1A1 protein expression in differentiating keratinocytes, visualized by Western blotting (Fig. 6). CYP1A1 protein was undetectable in control cultures differentiated for 6 days in the presence of vehicle, resveratrol, or α-naphthoflavone. The TCDD-induced CYP1A1 protein expression was nearly completely blocked at day 2 and day 4 in TCDD- and resveratrol-cotreated cultures (Fig. 6). This cotreatment was less efficacious at day 6, and this might be explained by time-dependent changes in the cellular levels and activities of AhR or AhR regulatory proteins and the half-life of CYP1A1 (Harper et al., 2006).

We studied whether resveratrol would also block TCDD-induced TGM mRNA expression, using the same conditions in which resveratrol effectively blocked TCDD-induced CYP1A1 expression at day 2 and day 4. In this study, TCDD induced maximal (8-fold) increases in TGM1 mRNA at day 4, and cotreatment with resveratrol completely blocked this effect of TCDD at day 4 (Fig. 5D). This result suggests AhR mediates TCDD-induced TGM1 expression. Resveratrol

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alone had the unexpected effect to induce higher TGM1 mRNA expression than TCDD alone (22-fold at day 2 and 156-fold at day 4 versus vehicle), even though it had no ability to induce AhR target gene expression under the same conditions (i.e., no CYP1A1 protein detected in Fig. 6).

Some similarities were observed in the regulation of other TGM isoforms. The maximal (5-fold) TCDD-induced increase in TGM3 mRNA occurred at day 4, and cotreatment with resveratrol completely blocked this effect of TCDD at day 4 (Fig. 5E). In contrast, maximal (5-fold) TCDD-induced increases in TGM5 mRNA also occurred at day 4, but cotreatment with resveratrol did not block the effect of TCDD on TGM5 mRNA (Fig. 5F). Treatment with resveratrol alone induced prematurely expression of TGM3 (227-fold at day 2; 18-fold at day 4) and TGM5 (194-fold at day 4) mRNA, compared with vehicle controls. It is not possible to evaluate the relative contributions of TGM1, TGM3, and TGM5 toward keratinocyte cross-linking activities since TGM substrates are not isoform-specific. Compared with TGM1, maximal TGM3 and TGM5 mRNA levels normally occur much later (>10 days) during in vitro differentiation (data not shown). Hence, it is not clear whether these early, initial changes in TGM3 and TGM5 mRNA levels are indicative of physiological regulation occurring later, in more terminally differentiated (granular) cells.

Results of these studies show that differentiating human keratinocyte cultures express all three TGM isoforms implicated in epidermal differentiation in vivo, that TGM1 is the dominant isoform (based on relative mRNA expression levels) probably responsible for the bulk of the cross-linking activities measured in situ, and that AhR may mediate TCDD-induced TGM expression and function in human skin keratinocytes.

**TCDD-Induced Changes in Keratinocyte Nuclear AP1 Protein Levels.** The lack of xenobiotic response elements in the TGM1 promoter invokes indirect mechanisms explaining TCDD actions in epidermal cells. One possibility is TCDD mediates signals that modulate intermediary factors involved in controlling epidermal TGM transcription and/or post-translational processing, ultimately leading to enhanced intracellular cross-linking and epidermal hyperkeratinization. Activator protein 1 subunits might be intermediary factors since epidermal expression of TGM1 in vivo and in vitro requires functional AP1 sites in the human TGM1 distal promoter (Phillips et al., 2004). Furthermore, genes encoding AP1 subunits (i.e., junD and c-jun) contain functional xenobiotic response elements, potential downstream targets of AhR (Hoffer et al., 1996).

We performed Western blot analyses to determine which AP1 proteins are present in nuclei of differentiating epidermal cells, since this has not been reported previously for cultures derived from normal tissue under conditions in which differentiation state is manipulated and monitored. We also determined how nuclear AP1 protein levels change in response to TCDD, with respect to TCDD concentration and duration of treatment (differentiation state).

Figure 7 shows representative results, but we evaluated and compared results from multiple blots and exposure times. Effects on nuclear translocation could not be discerned due to nonspecific interactions of the antibodies with cytoplasmic proteins. A low level of Fos B immunoreactivity was detected in nuclei of proliferating (day 0) and early differentiating (day 2 vehicle) cultures. Fos B levels tended to be greater in TCDD-treated cells at day 2, but after day 2 no signal was detected regardless of treatment. c-Fos immunoreactivity was low or undetectable in proliferating cells and exhibited biphasic up-regulation during in vitro differentiation, achieving maximal levels at day 4. The response to TCDD also seemed biphasic: c-Fos protein levels tended to be lower at day 4, but greater at day 6 (versus vehicle). Nuclear Fra-1 and Fra-2 localized preferentially to proliferating and differentiating cells, respectively. Both were modestly increased in nuclei of TCDD-treated cells (versus vehicle), depending on the concentration and duration of TCDD exposure. This was more evident at shorter exposure times (data not shown). Jun B protein levels tended to increase in response to TCDD, particularly at early time points during in vitro differentiation. Activator protein 1 subunits might be intermediary factors since epidermal expression of TGM1 in vivo and in vitro requires functional AP1 sites in the human TGM1 distal promoter (Phillips et al., 2004). Furthermore, genes encoding AP1 subunits (i.e., junD and c-jun) contain functional xenobiotic response elements, potential downstream targets of AhR (Hoffer et al., 1996).
vitro differentiation. Jun D protein levels tended to decrease during in vitro differentiation and were largely unaffected by TCDD. Ambiguous results were obtained for c-Jun blots due to nonspecific interactions (data not shown).

These study results identified three variables affecting nuclear levels of individual AP1 subunits: differentiation-specific factors and the concentration and duration of TCDD exposure. They suggest that TCDD exposure modulates nuclear AP1 protein levels in differentiating epidermal cells. Further studies are needed to determine whether TCDD exposure leads to AP1-mediated transcriptional activation of TGM1 or other target genes and whether post-transcriptional or post-translational mechanisms also contribute toward the increased TGM catalysis and the hyperkeratinized phenotype of TCDD-exposed human skin.

**Discussion**

Epidermal cells express many of the 57 functional human CYP genes, including CYP1–4 genes responsible for metabolism of most commonly prescribed drugs (Guengerich, 2006). This is the first quantitative study demonstrating interactions between cellular differentiation state and drug-induced changes in cyp expression in human epidermal cells. Retinoic acid and classic aryl hydrocarbon receptor ligands modulate epidermal differentiation (Fisher and Voorhees, 1996; Loertscher et al., 2001), and they are potent modulators of epidermal CYP1–4 expression. Responses to these receptor ligands conform to general rules. If the CYP gene is strongly regulated by differentiation-specific factors, drug challenge depressed epidermal mRNA expression. If the CYP gene is weakly (or not) regulated by differentiation-specific factors, drug exposure either augmented or had little effect on mRNA expression.

Skin is the most frequently reported target of adverse drug reactions (Roujeau, 2005), necessitating an understanding of how drug exposure affects epidermal P450 expression, drug metabolism, and disposition. Experimentally, pharmacokinetic variables make it difficult to predict the drug exposure time required to effect maximal P450 induction or inhibition (e.g., drug stability, half-life, and metabolic fate) (Tanaka, 1998). We treated differentiating keratinocytes for 6 days because this time was required for keratinocytes to transition from basal (proliferative) to spinous (differentiating) phenotypes in vitro. Earlier or transient drug-induced changes in P450 mRNA levels were not measured. Drug exposure can induce enzyme activities that accelerate drug disposition, thereby lowering effective drug concentrations. These considerations may influence why we measured no significant changes in P450 expression is response to miconazole, rifampicin, dexamethasone, and dapsone. Under different assay conditions, any one of these drugs may be a potent modulator of epidermal P450s, including P450s not included in these studies.

Miconazole, rifampicin, dexamethasone, and dapsone are nevertheless representative of drugs currently used in dermatology practice. Cytochrome P450 enzymes (e.g., CYP2C9 and 2E1) bioactivate dapsone and sulfonamides (e.g., sulfamethoxazole). This catalysis generates reactive nitrogen species that form covalent adducts with keratinocyte proteins (Wolf et al., 2000; Roychowdhury et al., 2005). These haptenized proteins can induce allergic drug reactions in human skin, especially in immunocompromised individuals (Merk et al., 2004; Roychowdhury et al., 2005). Azole antifungal agents such as miconazole are cytochrome P450 inducers or inhibitors, depending on concentration. Rifampicin is a potent P450 inducer and broad-spectrum antimicrobial (Tsankov and Angelova, 2003; Parkinson et al., 2004).

Exposure to β-naphthoflavone and TCDD during in vitro differentiation induced morphological changes reminiscent of chloracne—pronounced hyperkeratinization of keratinocytes lining sebaceous follicles in human skin (Crow and Puhvel, 1991). TGM cross-linking mediates the hyperkeratinized phenotype in chloracne and in TCDD-exposed keratinocyte cultures (Puhvel et al., 1984; Berkers et al., 1995). TGM protein substrates include cytokeatin and cornified cell envelope precursor proteins. Hyperkeratinization is most pronounced in more terminally differentiated keratinocyte cultures, consistent with the ability of TCDD to activate AhR more effectively in differentiating keratinocytes (Swanson, 2004). During differentiation, keratinocytes acquire regulatory cofactors (e.g., calcium, nucleotides) in their cellular milieu essential for post-translational processing and catalytic activation of expressed TGM proteins; this occurs mainly in the outer epidermal layers (Gibson et al., 1996).

Retinoids modulate epidermal differentiation, and AhR-dependent modulation of retinoid-metabolizing enzymes may explain some of the cutaneous manifestations of TCDD exposure (Fisher and Voorhees, 1996; Yamamoto and Tokura, 2003). The liver fibrosis and altered retinoid levels in AhR null mice were attributed to decreased hepatic CYP2C39 expression, a retinoic acid 4-hydroxylase (Andreola et al., 2004). Oxygenated retinoid metabolites have demonstrable biological activities in keratinocytes (Baron et al., 2005). Four P450s (CYP2C18, 3A4, 2C9, and 2S1) in this study have 4-hydroxylase activities toward retinoic acid (Marill et al., 2004; Baron et al., 2005; Saarikoski et al., 2005). Of these, CYP2S1 mRNA was the most abundant, the only P450 upregulated by retinoic acid exposure and not regulated by TCDD exposure. Retinoids also induce CYP2S1 in human skin (Saarikoski et al., 2005). It is not apparent why TCDD failed to substantially induce CYP2S1 in differentiating epidermal cultures since TCDD strongly induces CYP2S1 expression in hepatoma cell cultures (Saarikoski et al., 2005). In contrast, coal tar induced CYP2S1 in human skin but only in one-half of treated individuals. It would be important to know whether the AhR null mutation alters CYP2S1 expression and function in the epidermis and other epithelia where it is relatively highly expressed. Additional studies are needed to elucidate the importance of cutaneous CYP2S1 in endogenous (or therapeutic) retinoid metabolism, epidermal barrier functions, and susceptibility to skin tumorigenesis.

The TGMs involved in cornified cell envelope formation have been elucidated relatively recently (Lorand and Graham, 2003); we understand poorly the transcriptional and post-translational mechanisms regulating their activities. This is the first quantitative study reporting time-dependent changes in TGM1, -3, and -5 mRNA levels during in vitro differentiation and direct evidence that TCDD induces TGM mRNA expression. Studies showed that TCDD induces TGM cross-linking and epidermal cornification in mouse skin and human epidermal cultures (Puhvel et al., 1984; Berkers et al., 1995), but they provided no evidence whether this involves transcriptional and/or post-translational mechanisms.
Other cutaneous effects of TCDD are controversial, but a consensus emerges that TCDD causes abnormal epidermal differentiation. This is evidenced in these studies by altered expression of CYP and differentiation-specific genes and in humans by aceneform cutaneous eruptions (chloracne) caused by dioxin poisoning (Crow and Puhvel, 1991; Yamamoto and Tokura, 2003).

A transcriptional mechanism could explain the TCDD-induced TGM expression, but TCDD may also modulate TGM post-translational processing and cellular levels of catalytic cofactors. In Hepa-1 cells, dioxin induced sustained increases in intracellular calcium (Puga et al., 1997). This effect should augment TGM catalysis in keratinocytes. In mouse fibroblasts, which express mainly TGM2, hydrogen peroxide stimulated TGM cross-linking (Lee et al., 2003). This is relevant because TCDD generates oxidative stress in some systems, and the oxidative stress-responsive Nr2f2 transcription factor is a downstream AhR target (Bock and Kohle, 2006; Kohle and Bock, 2006). Keratinocyte TGM activities were unaffected by substituting hydrogen peroxide for TCDD in our system (versus vehicle; data not shown).

The 10-fold increases in keratinocyte TGM1 expression and cross-linking are comparable with the -fold changes reported for most differentially expressed genes in TCDD-treated SIK cells, derived from human epidermal keratinocytes (Rea et al., 2002). TGM1 was not a differentially expressed gene in this microarray study, possibly due to differences in TCDD exposure time and differentiation state (10-day TCDD exposure; SIK cultures at 80% confluence). In confluent cultures derived from normal human epidermis, acute (hours) TCDD exposure induced matrix metalloproteinase-1 expression; this response was mediated by functional AP1 sites in the matrix metalloproteinase-1 gene (Murphy et al., 2004). The effects of TCDD on TGM mRNA developed over days and may be partly mediated by interactions of AP1 proteins with TGM1 or other regulatory genes.

An over-riding conclusion is differentiation state can profoundly influence how epidermal cells respond to environmental agents, affecting metabolism of drugs, toxins, and endogenous ligands. P450 expression and differentiated keratinocyte functions (e.g., cornified cell envelope formation) are integrally linked. Activator protein 1 proteins regulate a large number of late differentiation genes in epidermal cells (Angel et al., 2001), and are probable intermediaries affecting P450 and TGM function in response to TCDD exposure. Regulation by AP1-DNA complexes is modulated by cellular levels of discrete AP1 subunits and by their interactions with other transcriptional regulators and regulatory elements existing in the cellular milieu (Rossi et al., 1998). Specific AP1 subunits may activate different effector pathways in different epidermal cell layers (differentiation states); this could result in modulation of TGM1 gene transcription in one state and TGM post-translational processing in another state.

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