

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Alters the Differentiation Pattern of Human Keratinocytes in Organotypic Culture

J. A. Loertscher,* C. A. Sattler,† and B. L. Allen-Hoffmann‡

‡Department of Pathology and †McArdle Laboratory for Cancer Research, University of Wisconsin Medical School, Madison, Wisconsin 53706; and *Environmental Toxicology Center, University of Wisconsin, Madison, Wisconsin 53706

Received December 29, 2000; accepted April 11, 2001

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Alters the Differentiation Pattern of Human Keratinocytes in Organotypic Culture. Loertscher, J. A., Sattler, C. A., and Allen-Hoffmann, B. L. (2001). *Toxicol. Appl. Pharmacol.* 175, 121–129.

Human exposure to the environmental toxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) produces a severe skin pathology known as chloracne. In these studies we employed a three-dimensional, organotypic model system to study the effects of TCDD on human skin. This model uses the spontaneously immortalized human keratinocyte cell line NIKS and recapitulates both the three-dimensional microenvironment and epithelial–mesenchymal interactions found in intact human skin. Treatment of the organotypic cultures with TCDD causes alterations in the pattern of keratinocyte terminal differentiation. Analysis at both the light and electron microscope levels reveals a fully differentiated cornified layer in TCDD-treated organotypic cultures at earlier time points than observed in vehicle (dimethyl sulfoxide)-treated controls. Furthermore, TCDD-treated organotypic cultures exhibit aberrant distribution of several differentiation-specific protein markers. Basal cells in TCDD- and DMSO-treated organotypic cultures show no differences in proliferation as measured by quantification of 5-bromo-2'-deoxyuridine (BrdU)-positive nuclei. No aberrant BrdU uptake was detected outside of the basal layer. Neither TUNEL labeling nor immunohistochemical staining with an antibody to active caspase-3 revealed increased apoptosis in TCDD-treated organotypic cultures relative to controls. These data clearly indicate that TCDD modulates homeostasis in a model of human stratifying epithelium independent of changes in proliferation and apoptosis, exclusively by impacting keratinocyte terminal differentiation. This TCDD-induced effect on differentiation-specific proteins results in profound changes in the tissue architecture. © 2001 Academic Press

Key Words: TCDD; keratinocytes; organotypic culture; NIKS.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is one congener of a family of halogenated aromatic hydrocarbons known as dioxins (reviewed in Safe, 1990; Van den Berg *et al.*, 1998). Dioxins are ubiquitous environmental toxins whose chemical stability and lipophilicity make them highly persistent in the environment and in biological systems. Human exposure to

TCDD produces an array of clinical manifestations. Chloracne, a hyperkeratotic skin disorder, is the most consistently observed pathology in exposed humans. Visual examination of children and adolescents exposed to relatively high levels of TCDD after a 1976 accident at a chemical plant near Seveso, Italy, has provided a detailed description of chloracne (Bertazzi *et al.*, 1998; Caputo *et al.*, 1988). Clinical observations from the Seveso accident coupled with other studies, including examinations of exposed pesticide plant workers (Moses and Prioleau, 1985), have led physicians to conclude that dermatopathology is the most reliable indicator of TCDD exposure in humans (reviewed in Dunigan, 1984). To date, histological examination of TCDD-exposed human skin has been limited and no examination of differentiation-specific proteins has been performed.

Skin is a continually renewing tissue that acts as one of the body's primary defense systems against environmental insult and often comes into contact with a wide spectrum of toxins. Skin is composed of two layers, the dermis and the epidermis. The dermis, whose primary cellular components are fibroblasts, forms the foundation upon which the epidermis lies. Dermal fibroblasts, the primary cellular component of the dermis, exist in a sea of extracellular matrix. These matrix components not only physically support the overlying layers but are also involved in biochemical signaling pathways (reviewed in Clark and Brugge, 1995). In addition to secreting matrix components, fibroblasts also secrete a spectrum of growth factors, which act on the overlying epidermis (Maas-Szabowski and Fusenig, 1996; Smola *et al.*, 1993). The epidermis is a stratified squamous epithelium composed primarily of keratinocytes. Keratinocytes within the epidermis are organized into four layers based upon morphological and biochemical properties: the basal, spinous, granular, and cornified layers (reviewed in Eckert *et al.*, 1997). The basal layer rests atop the dermis and is in direct contact with the specialized extracellular matrix proteins of the basement membrane. Basal keratinocytes have the ability to replicate and are the source of all suprabasal keratinocytes within the epidermis. As basal cells divide, certain daughter cells lose contact with the basement membrane. These cells assume a suprabasal position and

continue movement upward through the layers of the epidermis until they enucleate and are sloughed from the surface of the skin, a process known as terminal differentiation. The differentiation process in keratinocytes is accompanied by a pattern of characteristic changes in gene expression; a number of proteins serve as markers of terminal differentiation. Undifferentiated basal keratinocytes express the keratins K5 and K14, while differentiating cells express K1 and K10. Other proteins that are expressed in a specific pattern during keratinocyte terminal differentiation include precursors of the cornified envelope such as involucrin, filaggrin, pancornulin, cornifin, loricrin, and elafin, as well as the cross-linking enzyme epidermal transglutaminase (Morley and Lane, 1994; Simon, 1994; Steinert and Marekov, 1995).

Historically, the effect of TCDD on keratinocytes has been studied *in vitro* using a monolayer culturing system (reviewed in Leigh, 1994). Although several laboratories have previously reported on the cellular responses of normal human or murine keratinocytes to TCDD *in vitro*, little evidence has been available to link these observations to the specific pathology observed in human skin. Traditional monolayer culturing techniques, while allowing for the elucidation of many of the biochemical and molecular mechanisms relating to TCDD toxicity, are unable to answer complex questions pertaining to the biology of tissues exposed to TCDD. The three-dimensional structure produced in the organotypic culturing system allows for examination of tissue morphology and differentiation characteristics *in vitro*. Furthermore, keratinocytes cultivated in organotypic culture are grown in the presence of fibroblasts, providing the opportunity to explore the role of epithelial-mesenchymal interactions in TCDD-induced dermatopathology.

Our studies introduce a novel system for examination of the effects of TCDD on human skin. Using an organotypic coculture system containing primary human fibroblasts and a near-diploid, immortalized human keratinocyte cell line, NIKS (Allen-Hoffmann, 2000), we show that TCDD causes an altered differentiation pattern, but no change in 5-bromo-2'-deoxyuridine (BrdU) uptake or markers of apoptosis. This model is a powerful tool that will be essential in future studies, not only to elucidate the mechanism by which TCDD causes accelerated differentiation, but also as a model system to evaluate the effects of xenobiotics on human skin.

MATERIALS AND METHODS

Cell treatments. TCDD (Wellington Laboratories, Guelph, Ontario, Canada) was prepared in dimethyl sulfoxide (DMSO) such that the final concentration of DMSO in the treatment medium did not exceed 0.1%. The final concentration of TCDD in the culturing medium was 10^{-8} M. Solvent controls included 0.1% DMSO.

Organotypic culture. Modification in the three-dimensional organotypic culture (Parenteau *et al.*, 1992) of keratinocytes has been previously described (Allen-Hoffmann, 2000). Briefly, a dermal equivalent was formed by mixing 75,000 normal human neonatal fibroblasts with type I collagen (Organogenesis, Canton, MA) in Ham's F-12 supplemented with 10% Fetal Clone II

(HyClone, Logan, UT) and 100 units penicillin and 100 μ g/ml streptomycin (P/S). The gel was allowed to contract before 300,000 NIKS cells were plated onto the collagen base. After 2 h, plating media was added and cultures were treated in the media with either TCDD or DMSO. Plating media is a mixture of Ham's F-12:Dulbecco's modified Eagle's medium (3:1, 1.88 mM calcium) supplemented with 0.2% Fetal Clone II, 0.4 μ g/ml hydrocortisone, 8.4 ng/ml cholera toxin, 5 mg/ml insulin, 24 mg/ml adenine, and P/S. On day 4, cultures were lifted to the air interface with sterile cotton pads and switched to cornification media. Cornification media is a mixture of Ham's F-12:Dulbecco's modified Eagle's medium (3:1, 1.88 mM calcium) supplemented with 2.0% Fetal Clone II, 0.4 μ g/ml hydrocortisone, 8.4 ng/ml cholera toxin, 5 mg/ml insulin, 24 mg/ml adenine, and P/S. Cultures were retreated at each media change. For proliferation assays, BrdU (Sigma, St. Louis, MO) was added to culture media at a final concentration of 10 μ M for 8 h prior to harvesting.

At 8 days postplating, cultures were either cryopreserved or fixed and paraffin embedded. Paraffin-embedded samples were fixed overnight in 4% paraformaldehyde phosphate-buffered saline, pH 7.2 (PBS), embedded in paraffin, and sectioned by the Pathology Department Histology Laboratory (University of Wisconsin-Madison, Madison, WI). For cryopreserved samples tissue was fixed for 2 h with 1% paraformaldehyde/PBS and stored in 20% sucrose/PBS overnight at 4°C in preparation for freezing. The tissue was then frozen in OCT media in liquid-chilled isopentane stored at -20°C and mounted on charged glass slides. Some paraffin-embedded sections were stained with hematoxylin and eosin for evaluation of culture morphology. Stained sections were viewed and video images were captured using an Olympus IX-70 microscope.

Electron microscopy. Fixed organotypic cultures were washed three times with 0.1 M cacodylate buffer, pH 7.4. Under a dissecting microscope, a scalpel was used to detach the polyester mesh supporting the organotypic culture from the plastic insert. The organotypic culture was cut with a scalpel into approximately 2 mm \times 4 mm pieces, which were stored overnight in 0.1 M cacodylate buffer, pH 7.4 buffer. Following postfixation with 1% osmium tetroxide at 4°C, the organotypic cultures were washed four times, 15 min each, with 0.1 M maleic acid, pH 6.5, before *en bloc* staining with 2% aqueous uranyl acetate for 1 h. After washing with distilled water, organotypic cultures were dehydrated with increasing concentrations of ethanol, 100% propylene oxide, and infiltrated with 1:1 propylene oxide:Eponate overnight. Organotypic cultures were embedded in fresh Eponate in flat embedding molds and oriented so they could be sectioned perpendicularly on a Reichert Ultracut E3 ultramicrotome equipped with a diamond knife. Thin sections were stained with lead citrate and examined in a Hitachi H-7000 electron microscope (Hitachi, San Jose, CA) operated at 75 kV.

Immunohistochemical analysis of organotypic cultures. Primary antibodies used were anti-transglutaminase-1 and anti-filaggrin (Biomedical Technologies Inc., Stoughton, MA), anti-keratin-1 (Novo Castra, Newcastle upon Tyne, UK), anti-involucrin (Sheibani, 1994), and anti-active caspase-3 (R and D Systems, Minneapolis, MN). For filaggrin and keratin-1 immunohistochemistry, paraffin-embedded organotypic cultures were serially sectioned (5 μ m), mounted on glass slides, and deparaffinized in xylene followed by an ethanol series. For transglutaminase-1 and involucrin immunohistochemistry, cryopreserved samples were sectioned, mounted on glass slides, and fixed for 5 min in ice-cold acetone. Sections to be stained with keratin-1 and filaggrin were microwaved in 10 mM sodium citrate, pH 6.0, for 3.5 min to promote antigen exposure. All sections were washed with PBS and blocked with 3% normal goat serum (Sigma) in PBS. Sections were incubated with primary antibody (1:100 anti-transglutaminase-1 for 1 h at 37°C; 1:25 anti-filaggrin for 1 h at room temperature; 1:20 anti-keratin-1 for 1 h at room temperature; 1:1000 anti-involucrin for 1 h at room temperature; and 1:50 anti-active caspase-3 overnight at 4°C). Sections were then incubated with secondary antibodies, a 1:500 dilution of Alexa 594-conjugated goat anti-rabbit IgG (for involucrin and active caspase-3), or Alexa 488-conjugated goat anti-mouse IgG (for all others) (Molecular Probes, Eugene OR). All sections were counterstained with 5 μ g/ml Hoechst 33258. Samples were viewed with an IX-70 inverted fluo-

rescence microscope (Olympus) equipped with FITC (470 ± 20 nm), Hoechst (525 ± 20 nm), and Texas Red (360 ± 20 nm) band-pass filters.

BrdU-labeled cultures were sectioned ($5 \mu\text{m}$), deparaffinized, and microwaved in 10 mM sodium citrate, pH 6.0, for antigen exposure. Sections were rinsed in PBS and peroxidase-quenched with 1:9 30% H_2O_2 :MeOH for 10 min. Sections were then treated with 2 N HCl for 20 min. After blocking with 5% normal goat serum/PBS, sections were incubated with anti-BrdU antibody (Becton Dickinson, San Jose, CA) for 3 h at room temperature. A biotinylated horse anti-mouse IgG was used, followed by the Vectastain Elite ABC reagent kit (Vector Laboratories, Burlingame, CA). Bound enzyme-linked biotin was detected with 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and 0.003% H_2O_2 /TBS.

In situ 3' end labeling of organotypic cultures. Free 3' ends of DNA were detected in sections of organotypic cultures ($5 \mu\text{m}$) using the APOPTAG Fluorescein *In Situ* Apoptosis Detection kit (Intergen, Purchase, NY). Sections were deparaffinized and protein digested with $20 \mu\text{g}/\text{ml}$ proteinase K for 15 min in water. Terminal deoxynucleotidyl transferase (TdT) in sample buffer was applied. Samples were covered with plastic coverslips and incubated for 1 h at 37°C . Slides were placed in 37°C stop buffer for 30 min. Antidigoxin fluor was applied in block buffer. Samples were covered with plastic coverslips and incubated 1 h at 37°C . Samples were counterstained with Hoechst 33258. Sections were viewed with an IX-70 inverted fluorescence microscope (Olympus) equipped with FITC (470 ± 20 nm) and Hoechst (525 ± 20 nm) band-pass filters.

RESULTS

TCDD Causes Accelerated Onset of Terminal Differentiation in Organotypic Culture

Organotypic coculture of human keratinocytes and dermal fibroblasts provides a powerful *in vitro* system in which to investigate TCDD-induced dermatopathology. Unlike monolayer culture of keratinocytes, which has traditionally been employed to explore the effects of TCDD on human skin, organotypic culture recapitulates the three-dimensional tissue microenvironment of skin and accounts for epithelial–mesenchymal interactions. Studies in both humans and rodents have indicated involvement of the dermis in the TCDD-induced dermatopathology, chloracne (Caputo *et al.*, 1988; Moses and Prioleau, 1985; Panteleyev *et al.*, 1997; Poland and Knutson, 1982). For example, in addition to epidermal hyperplasia, hair follicle abnormalities and dermal cyst formation are cited as hallmarks of chloracne. Therefore, any critical examination of the effects of TCDD on skin should account for both dermal and epidermal compartments. The system described here provides a novel method to explore the impact of TCDD on stratified squamous epithelium, which reveals the full spectrum of the effects of TCDD on human skin.

Organotypic cultures of keratinocytes treated with TCDD in the medium showed changes in morphology and differentiation characteristics as compared to vehicle controls. At 8 days postplating, TCDD-treated organotypic cultures possessed a well-developed cornified layer that was absent in time-matched controls (Fig. 1). This effect was also observed in organotypic cultures of primary human keratinocytes (data not shown), verifying that NIKS cells in organotypic culture responded to TCDD in a manner identical to that observed in primary human keratinocytes. Analysis of TCDD-treated cultures using electron microscopy at 11 days postplating revealed definitive

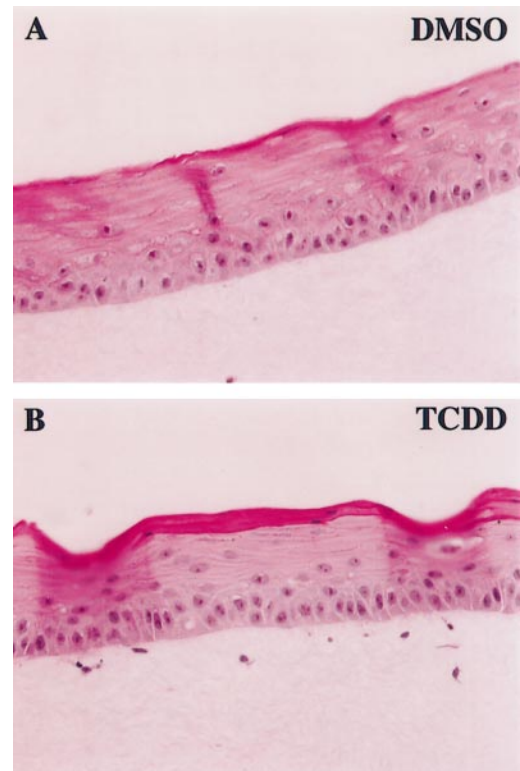
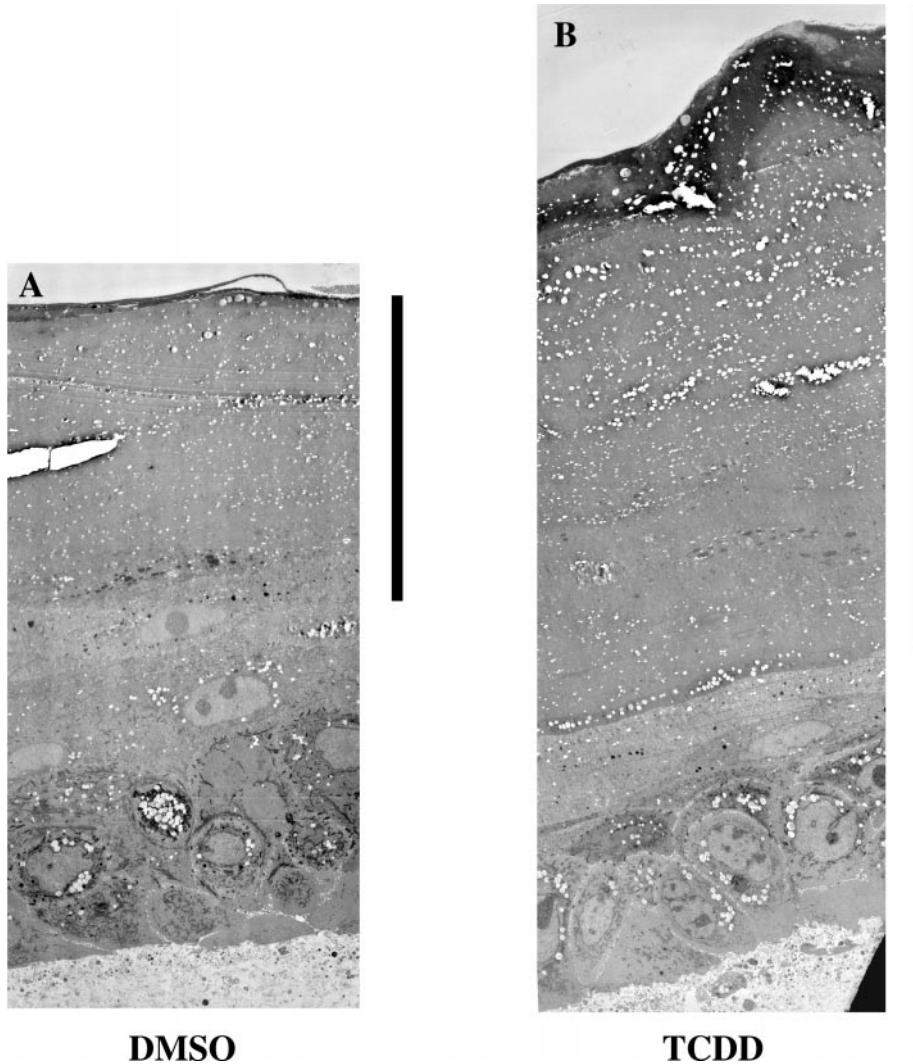


FIG. 1. TCDD causes early onset of terminal differentiation in keratinocytes in organotypic culture. NIKS cells were grown in organotypic culture in the presence of (A) DMSO or (B) TCDD for 8 days. Cultures were then fixed, paraffin embedded, sectioned, and stained with hematoxylin and eosin. Original magnification $400\times$.

signs of accelerated differentiation (Fig. 2). In the immediately suprabasal layers of control samples, keratinocytes were only beginning to flatten, whereas these same cells in TCDD-treated cultures displayed the flattened appearance of more mature keratinocytes. Furthermore, TCDD-treated samples exhibited a layer of keratinized tissue far more extensive than that observed in vehicle control cultures. Measurements taken from the electron micrographs showed that the ratio of the thickness of the keratinized to nonkeratinized layers was substantially greater in the TCDD-treated cultures (Table 1). This increased ratio can be attributed to both an increase in the amount of keratinized material and a flattening of the nonkeratinized spinous layer in the TCDD-treated cultures. These observations are important not only because the organotypic culturing system provides a model in which the effect of TCDD on skin can be studied *in vitro*, but also because the effect produced in the model resembles the hyperkeratotic characteristics of chloracne in intact human skin.

TCDD Causes Aberrant Expression of Differentiation-Specific Protein Markers

In addition to observing gross morphological changes in the TCDD-treated organotypic cultures, we also assessed whether



DMSO

TCDD

FIG. 2. Morphological changes are visible at the electron microscopy level. Electron micrographs of (A) DMSO-treated organotypic cultures and (B) TCDD-treated organotypic cultures at 11 days postplating show increased keratinization and flattening of the spinous and granular layers in TCDD-treated samples. Dark line indicates keratinized layer. Original magnification 1500 \times .

the cultures displayed any abnormalities in expression of differentiation-specific protein markers (Fig. 3). Organotypic cultures of both primary human keratinocytes and NIKS cells faithfully support patterns of differentiation marker expression nearly identical to those of intact skin (Clotfelter and Allen-Hoffmann, unpublished observations). TCDD-treated and ve-

TABLE 1

Ratio of Thickness of the Keratinized Layer to Nonkeratinized Layers of Organotypic Cultures as Measured by Electron Microscopy

| | Day 8 | Day 11 |
|------|-----------------|-----------------|
| DMSO | 0.20 \pm 0.03 | 0.57 \pm 0.18 |
| TCDD | 0.28 \pm 0.06 | 1.6 \pm 0.28 |

hicle control rafts were harvested 8 days postseeding and immunohistochemistry was performed to examine the expression and spatial distribution of filaggrin, involucrin, transglutaminase-1, and keratin 1 (Fig. 3). All markers examined were present in both TCDD-treated and control cultures. However, the pattern of differentiation marker expression was aberrant in TCDD-treated cultures. The expression pattern of filaggrin, an intermediate filament-associated protein, was most dramatically altered by TCDD treatment (Figs. 3A and 3B). Control samples exhibited characteristically punctate, but evenly distributed, staining in the granular layer. In contrast, filaggrin expression was evident in cellular layers more proximal to the basal layer in TCDD-treated samples than in control samples. Furthermore, filaggrin expression in TCDD-treated samples was patchy within the granular layer, with areas of very bright staining and areas of very faint staining, as opposed to the even

distribution observed in control sections. These two observations suggest that TCDD interferes with the regulation of both the temporal and spatial expression of filaggrin. The expression pattern of involucrin, a protein component of the cornified envelope, was altered in a manner similar to that observed with filaggrin. Involucrin expression was evident in many basal keratinocytes in TCDD-treated cultures, a phenomenon never observed in control cultures, where involucrin is found only in suprabasal cells (Figs. 3C and 3D). The more basal-proximal expression of filaggrin and involucrin proteins in TCDD-treated samples, like the morphological changes observed at the electron microscope level, indicates a compression of the spinous layer due to TCDD treatment. Additionally, irregular distribution of the membrane-associated differentiation marker transglutaminase-1 was found in some TCDD-treated cultures (Figs. 3E and 3F). Instead of staining in a crisp, contiguous ring at the cell membrane, as in control samples, the staining pattern of transglutaminase-1 in TCDD-treated cultures was somewhat irregular and disrupted at the cell membrane. Keratin 1 expression appeared unaltered by TCDD treatment (Figs. 3G and 3H). The overall enhanced differentiation is consistent with the initial histological observation that TCDD-treated organotypic cultures produce a cornified layer earlier than controls.

TCDD Does Not Cause a Change in BrdU Uptake in Keratinocytes in Organotypic Culture

Tissue homeostasis in skin relies on the balance among differentiation, proliferation, and death. In order to assess whether the accelerated differentiation we observed in TCDD-treated organotypic cultures was the result of an increase in the rate of proliferation or apoptosis, we performed BrdU uptake experiments, *in situ* DNA 3' end labeling, and immunohistochemical analysis for active caspase-3. We first sought to determine whether the accelerated differentiation observed could be attributed to changes in proliferation. At 8 days postplating, TCDD-treated and control organotypic cultures were given 10 $\mu\text{g}/\text{ml}$ BrdU in the media 8 h prior to harvesting. After harvesting, cultures were paraffin embedded, sectioned, and stained with an anti-BrdU antibody. The number of BrdU-positive nuclei was calculated as a percentage of the total number of nuclei. The number of BrdU-positive nuclei ranged from 4 to 15% but was not significantly different between TCDD-treated and control groups (Fig. 4). A *t* test analysis of the difference in percentage of BrdU-positive nuclei between treatment and control groups gave a *p* value of 0.92 at a 95% confidence level.

TCDD Does Not Induce Apoptosis in Keratinocytes in Organotypic Culture

Apoptosis is known to be involved in the modulation of both health and disease states in the skin and its appendages (reviewed in Haake and Polakowska, 1993; Raskin, 1997), indi-

cating that keratinocytes are fully equipped to initiate an apoptotic cascade in response to a variety of endogenous and exogenous stimuli. Biochemical analysis of keratinocytes has verified their ability to express functional caspases, the proteases responsible for regulating and executing the orderly breakdown of cellular structures observed during apoptosis (Weil *et al.*, 1999). Some researchers have suggested that keratinocyte terminal differentiation is a specialized form of apoptosis, however, recent studies have indicated that the two events are distinct instead of being interdependent (Gandarillas *et al.*, 1999; Mitra *et al.*, 1997; Weil *et al.*, 1999).

In situ DNA fragments were detected using TUNEL labeling. Sections from both treatment and control groups, harvested 8 days postplating, were prepared according to the manufacturer's protocol. Cycloheximide-treated positive controls displayed numerous positive nuclei in the basal, immediately suprabasal, and cornified layers. Those nuclei detected as positive by 3' DNA end-labeling also displayed morphological characteristics of apoptosis, seen by Hoechst counterstaining. In contrast, both TCDD-treated and vehicle controls showed a negligible amount of labeled cells in all cell layers except the cornified layer (Fig. 5). Immunohistochemical analysis for active caspase-3 showed very faint staining in the spinous and granular layers, with no increased staining in TCDD-treated cultures (Fig. 6). We therefore conclude that TCDD treatment does not aberrantly induce apoptosis in keratinocytes. These data strongly suggest that accelerated differentiation observed in TCDD-treated organotypic cultures is due to a change in a differentiation-specific pathway, which does not overlap with apoptotic pathways.

DISCUSSION

We found that TCDD treatment of organotypic cultures containing epidermal and dermal compartments alters the pattern of keratinocyte terminal differentiation. No changes in BrdU uptake or markers of apoptosis were observed. Treatment of a three-dimensional organotypic culturing system using NIKS keratinocytes and dermal fibroblasts definitively showed that TCDD causes premature flattening and cornification of suprabasal keratinocytes. All protein markers of differentiation examined were present in TCDD-treated organotypic cultures, as seen by immunohistochemistry, but were expressed in cellular layers more proximal to the basement membrane compared to vehicle controls. No difference in BrdU uptake was observed between treatment and control organotypic cultures. Both TUNEL analysis and immunohistochemical detection of active caspase-3 expression revealed no increase in apoptosis in TCDD-treated organotypic cultures compared to control cultures. We obtained similar results in monolayer cultures of keratinocytes (Loertscher *et al.*, 2001). We therefore conclude that TCDD causes accelerated differentiation in keratinocytes by a mechanism that is independent of apoptotic and proliferative pathways.

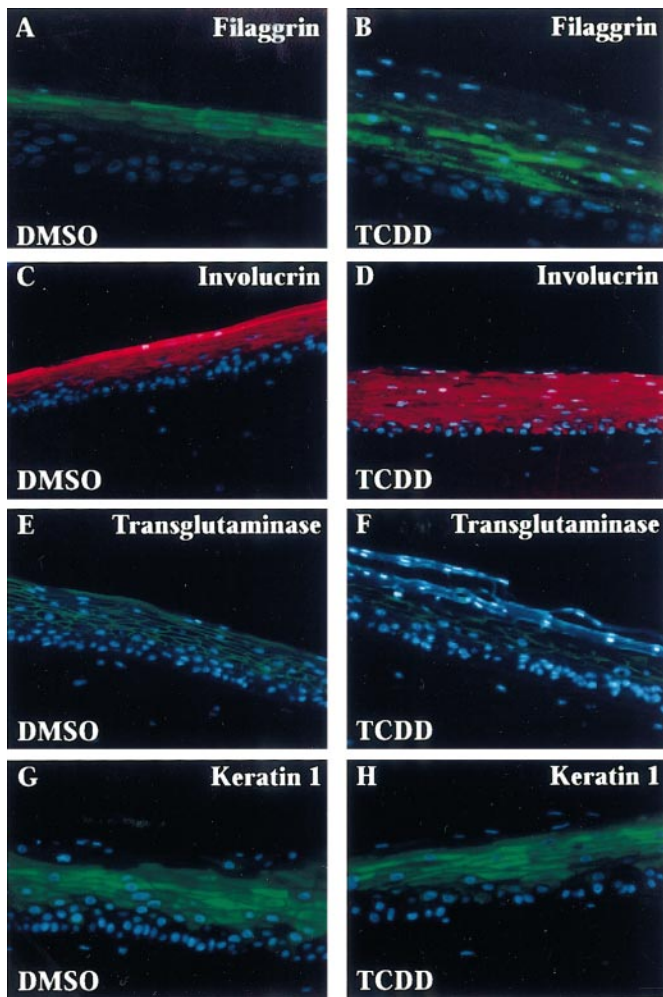


FIG. 3. Localization of differentiation markers is aberrant in TCDD-treated organotypic cultures. Organotypic cultures were treated with DMSO (A, C, E, and G) or TCDD (B, D, F, and H). Eight days postplating, cultures were fixed, sectioned, and stained with filaggrin (A and B), involucrin (C and D), transglutaminase-1 (E and F), or keratin 1 (G and H). (C, D, E, F) Original magnification 300 \times . (A, B, G, H) Original magnification 400 \times .

Our studies sought to elucidate the mechanism by which TCDD causes chloracne, a human skin pathology, through examination of the cell biological effects of TCDD on organotypic cultures containing both the primary epidermal and dermal cells found in human skin. Although the skin pathology caused by exposure of humans to TCDD has been well characterized, elucidation of the mechanism by which TCDD causes chloracne has been hindered by several factors. TCDD fails to produce chloracne in most laboratory species. Therefore, animal studies have been primarily limited to the hairless (*hr/hr*) mouse, which does display a chloracne phenotype (reviewed in Panteleyev *et al.*, 1998). Furthermore, a survey of 23 cell lines failed to identify a cell line of epithelial origin that could be used as a model to study TCDD-induced pathology (Knutson and Poland, 1980). All epithelial cell lines examined displayed no changes in morphology, viability, or growth upon

exposure to TCDD, leaving researchers without an adequate *in vitro* system in which to study TCDD-generated pathologies. Subsequent studies using primary human keratinocytes cultured in monolayer have been performed with equivocal results (Greenlee *et al.*, 1985; Hudson *et al.*, 1986; Milstone and LaVigne, 1984; Osborne and Greenlee, 1985), most likely reflecting the possible interplay between epidermis and dermis. Studies using cocultures of keratinocytes and dermal fibroblasts in the absence of TCDD have clearly demonstrated mutually inductive growth factor production by these two cell types. Mutual growth factor induction in cocultures impacts proliferation in both fibroblasts and keratinocytes and modulates epithelial architecture and protein expression (Maas-Szabowski *et al.*, 1999, 2000; Smola *et al.*, 1993). These results clearly illustrate the vital role of epithelial–mesenchymal interactions in the maintenance of tissue homeostasis and emphasize the importance of accounting for this relationship when exploring the impact of an exogenous agent on a tissue.

The aryl hydrocarbon receptor (AhR) signal transduction pathway is thought to mediate the toxic effects of TCDD and other polycyclic aromatic hydrocarbons (Poland and Glover, 1980). The AhR, a ligand-activated transcription factor, is a member of the basic helix–loop–helix per-arnt-sim (bHLH-PAS) family of transcription factors (reviewed in Hankinson, 1995; Schmidt and Bradfield, 1996). Although the fundamental AhR-dependent mechanism by which TCDD induces gene expression is well understood, it is not known how TCDD causes pathologies such as chloracne. The expression of a battery of toxicologically significant genes, including cyto-

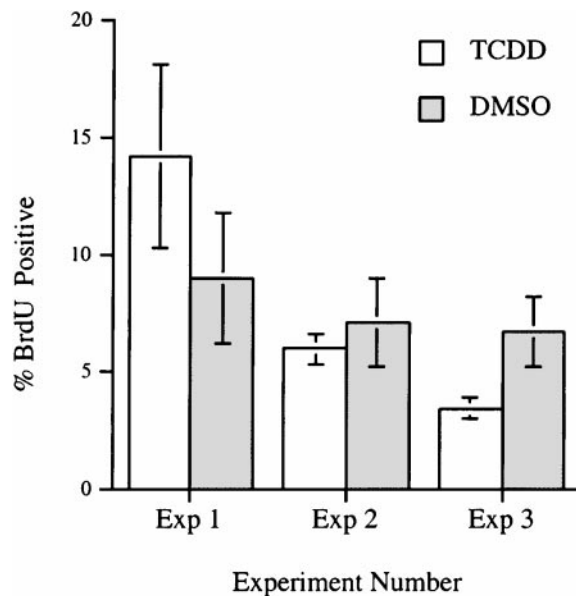


FIG. 4. Basal cell proliferation as measured by BrdU uptake is not affected by TCDD treatment. Organotypic cultures were treated for 8 days with DMSO (white bars) or TCDD (gray bars) and exposed to 10 $\mu\text{g}/\text{ml}$ BrdU on the day of harvest. BrdU-positive basal cells were counted. Three different experiments with standard error are depicted.

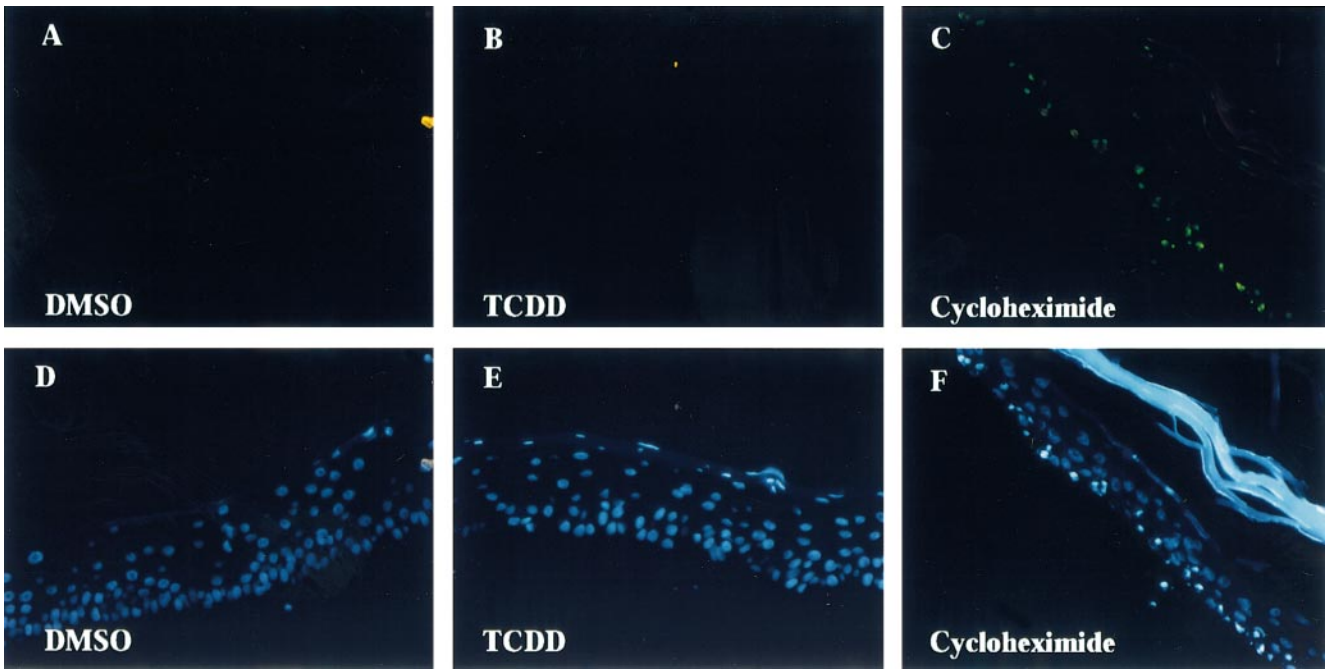


FIG. 5. TCDD does not induce nucleosomal fragmentation in keratinocytes in organotypic culture. Organotypic cultures were treated for 8 days with DMSO (A and D), TCDD (B and E), or 30 $\mu\text{g/ml}$ cycloheximide as a positive control (C and F). *In situ* DNA 3' end-labeling was performed on sectioned organotypic cultures and the fluorescent label was visualized (A–C). Sections were counterstained with Hoechst to visualize the nuclei (D–F).

chrome P450 1A1 (CYP1A1), cytochrome P450 1B1, glutathione *S*-transferase, and NADPH quinone oxidoreductase, is induced by TCDD via AhR (Prough, 1996); however it is

unknown if induction of these genes is causally linked to TCDD-generated pathologies. Genes independent of detoxification pathways, such as plasminogen activator inhibitor-2,

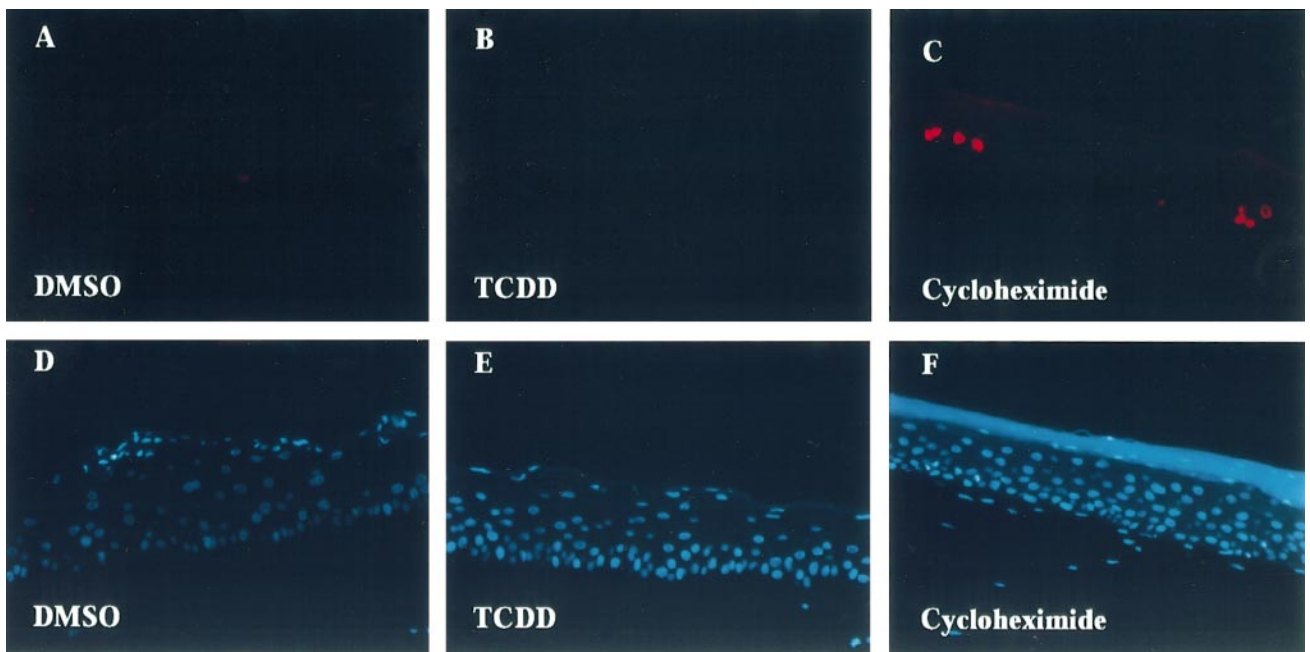


FIG. 6. TCDD does not induce protein expression of active caspase-3 in keratinocytes in organotypic culture. Organotypic cultures were treated for 8 days with DMSO (A and D), TCDD (B and E), or 5 $\mu\text{g/ml}$ cycloheximide as a positive control (C and F). Eight days postplating, cultures were fixed, paraffin embedded, and sectioned. Sections were stained using an antibody against active caspase-3 (A–C). Sections were counterstained with Hoechst to visualize the nuclei (D–F).

interleukin-1 β (Sutter *et al.*, 1991), and transforming growth factor- α (Choi *et al.*, 1991), have been identified as being TCDD responsive, suggesting that AhR may have physiological roles outside of xenobiotic metabolism (Dohr *et al.*, 1994). Furthermore, many members of the bHLH superfamily are known to be involved in growth and differentiation, leading to speculation that the AhR/Arnt dimer may play a role in skin differentiation. For example, Id-1, a HLH protein that lacks a basic DNA-binding domain, functions as a negative regulator of bHLH proteins. Id-1 overexpression in keratinocytes leads to decreased differentiation and an enhanced lifespan in culture (Alani *et al.*, 1999).

The role that the AhR plays in the TCDD-induced changes in organotypic cultures of human keratinocytes remains unknown. Research conducted in our laboratory has shown that CYP1A1 mRNA expression can be induced in an AhR-dependent manner in the absence of a xenobiotic, simply by altering the adhesive characteristics of the cells. For example, suspension of keratinocytes in a semisolid medium (Green, 1977) results in induction of CYP1A1 in the absence of a xenobiotic (Sadek and Allen-Hoffmann, 1994). Suspension, a manipulation that ablates both cell-cell and cell-substratum contacts, is a technique used to mimic the process of keratinocyte differentiation in which basal cells must modify their adhesive characteristics in order to migrate upward. Suspension triggers a variety of changes in gene expression parallel to the characteristic changes in gene expression observed during keratinocyte differentiation. The novel observation that genes classically associated with xenobiotic-metabolizing enzymes can be induced in the absence of a xenobiotic simply by modifying cellular adhesive characteristics is critical because it strongly suggests a role for AhR/Arnt signal transduction in not only detoxification but also normal physiological pathways. This dual role for AhR signal transduction in skin was originally proposed by Poland and Glover soon after AhR was identified as the mediator of TCDD toxicity (Poland and Glover, 1980). It is tempting to speculate that AhR, like other bHLH-PAS family members, plays a role in formation or maintenance of a three-dimensional tissue structure in skin. For example, the *Drosophila* protein tracheless regulates the transcription of an FGF family homolog called breathless. Both tracheless and breathless are required for complete formation of several epithelial tube structures, including the trachea, salivary ducts, and filzkoper (Glazer and Shilo, 1991; Isaac and Andrew, 1996; Wilk *et al.*, 1996). Based on the results of our studies, we speculate that the AhR regulates genes involved in squamous differentiation, resulting in a tissue that possesses high tensile strength and promotes barrier function.

In conclusion, we have shown that TCDD exerts effects on keratinocytes in organotypic culture by altering the differentiation program of these cells without changing apoptotic or proliferative pathways. We have introduced a three-dimensional system for the culture of a normal human keratinocyte cell line (NIKS), which can be used to assess the effects of a

xenobiotic agent, TCDD. Further experimentation using this system will allow us to determine whether the accelerated differentiation observed upon TCDD treatment is dependent on AhR in the dermal or epidermal compartments of the model. In addition to providing a model system in which to investigate the cellular and molecular biological basis of the pathological effects of TCDD, this organotypic culture model system introduces a general system for analyzing the effects of xenobiotics on skin.

ACKNOWLEDGMENTS

We extend our sincere thanks to Nancy Parenteau and Organogenesis for the kind gift of collagen type-1 used in organotypic cultures. We are grateful for the excellent preparation of histological samples by Toshi Kinoshita. Thanks to Dr. Mary Lindstrom for assistance with statistical analysis. We are especially thankful to all members of the Allen-Hoffmann Laboratory, particularly Cathy Ivarie for technical assistance with the organotypic cultures, and Michael Pickart and Justine Clotfelter for suggestions on immunohistochemical techniques.

REFERENCES

- Alani, R. M., Hasskarl, J., Grace, M., Hernandez, M. C., Israel, M. A., and Munger, K. (1999). immortalization of primary human keratinocytes by the helix-loop-helix protein, Id-1. *Proc. Natl. Acad. Sci. USA* **96**, 9637-9641.
- Allen-Hoffmann, B. L., Schlosser, S. J., Ivarie, C. A., Sattler, C. A., Meisner, L. F., and O'Connor, S. L. (2000). Normal growth and differentiation in a spontaneously immortalized near-diploid human keratinocyte cell line, NIKS. *J. Invest. Dermatol.* **114**, 444-455.
- Bertazzi, P. A., Bernucci, I., Brambilla, G., Consonni, D., and Pesatori, A. C. (1998). The Seveso studies on early and long-term effects of dioxin exposure: A review. *Environ. Health Perspect.* **106**(Suppl. 2), 625-633.
- Caputo, R., Monti, M., Ermacora, E., Carminati, G., Gelmetti, C., Gianotti, R., Gianni, E., and Puccinelli, V. (1988). Cutaneous manifestations of tetrachlorodibenzo-*p*-dioxin in children and adolescents. Follow-up 10 years after the Seveso, Italy, accident. *J. Am. Acad. Dermatol.* **19**, 812-819.
- Choi, E. J., Toscano, D. G., Ryan, J. A., Riedel, N., and Toscano, Jr., W. A. (1991). Dioxin induces transforming growth factor-alpha in human keratinocytes. *J. Biol. Chem.* **266**, 9591-9597.
- Clark, E. A., and Brugge J. S. (1995). Integrins and signal transduction pathways: The road taken. *Science* **268**, 233-239.
- Dohr, O., Vogel, C., and Abel, J. (1994). Modulation of growth factor expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Exp. Clin. Immunogenet.* **11**, 142-148.
- Dunigan, W. G. (1984). Cutaneous signs of systemic toxicity due to dioxins and related chemicals. *J. Am. Acad. Dermatol.* **10**, 688-700.
- Eckert, R. L., Crish, J. F., and Robinson, N. A. (1997). The epidermal keratinocyte as a model for the study of gene regulation and cell differentiation. *Physiol. Rev.* **77**, 397-424.
- Gandarillas, A., Goldsmith, L. A., Gschmeissner, S., Leigh, I. M., and Watt, F. M. (1999). Evidence that apoptosis and terminal differentiation of epidermal keratinocytes are distinct processes. *Exp. Dermatol.* **8**, 71-79.
- Glazer, L., and Shilo, B. Z. (1991). The *Drosophila* FGF-R homolog is expressed in the embryonic tracheal system and appears to be required for directed tracheal cell extension. *Genes Dev.* **5**, 697-705.

- Green, H. (1977). Terminal differentiation of cultured human epidermal cells. *Cell* **11**, 405–416.
- Greenlee, W. F., Dold, K. M., and Osborne, R. (1985). Actions of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on human epidermal keratinocytes in culture. *In Vitro Cell. Dev. Biol.* **21**, 509–512.
- Haake, A. R., and Polakowska, R. R. (1993). Cell death by apoptosis in epidermal biology. *J. Invest. Dermatol.* **101**, 107–112.
- Hankinson, O. (1995). The aryl hydrocarbon receptor complex. *Annu. Rev. Pharmacol. Toxicol.* **35**, 307–340.
- Hudson, L. G., Toscano, Jr., W. A., and Greenlee, W. F. (1986). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) modulates epidermal growth factor (EGF) binding to basal cells from a human keratinocyte cell line. *Toxicol. Appl. Pharmacol.* **82**, 481–492.
- Isaac, D. D., and Andrew, D. J. (1996). Tubulogenesis in *Drosophila*: A requirement for the tracheless gene product. *Genes Dev.* **10**, 103–117.
- Knutson, J. C., and Poland, A. (1980). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin: Failure to demonstrate toxicity in twenty-three cultured cell types. *Toxicol. Appl. Pharmacol.* **54**, 377–383.
- Leigh, I. M., and Watt, F. M. (1994). The culture of human epidermal keratinocytes. In *The Keratinocyte Handbook* (I. M. Leigh, E. B. Lane, and F. M. Watt, Eds.), pp. 43–52. Cambridge University Press, Cambridge.
- Loertscher, J. A., Sadek, C. S., and Allen-Hoffmann, B. L. (2001). Treatment of normal human keratinocytes with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin causes a reduction in cell number, but no increase in apoptosis. *Toxicol. Appl. Pharmacol.* **175**, 114–120.
- Maas-Szabowski, N., and Fusenig, N. E. (1996). Interleukin-1-induced growth factor expression in postmitotic and resting fibroblasts. *J. Invest. Dermatol.* **107**, 849–855.
- Maas-Szabowski, N., Shimotoyodome, A., and Fusenig, N. E. (1999). Keratinocyte growth regulation in fibroblast cocultures via a double paracrine mechanism. *J. Cell Sci.* **112**, 1843–1853.
- Maas-Szabowski, N., Stark, H. J., and Fusenig, N. E. (2000). Keratinocyte growth regulation in defined organotypic cultures through IL-1-induced keratinocyte growth factor expression in resting fibroblasts. *J. Invest. Dermatol.* **114**, 1075–1084.
- Milstone, L. M., and LaVigne, J. F. (1984). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin induces hyperplasia in confluent cultures of human keratinocytes. *J. Invest. Dermatol.* **82**, 532–534.
- Mitra, R. S., Wrona-Smith, T., Simonian, P., Foreman, K. E., Nunez, G., and Nickoloff, B. J. (1997). Apoptosis in keratinocytes is not dependent on induction of differentiation. *Lab. Invest.* **76**, 99–107.
- Morley, S. M., and Lane, E. B. (1994). The keratinocyte cytoskeleton. In *The Keratinocyte Handbook* (I. Leigh, B. Lane, and F. Watt, Eds.), pp. 293–321. Cambridge University Press, Cambridge.
- Moses, M., and Prioleau, P. G. (1985). Cutaneous histologic findings in chemical workers with and without chloracne with past exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J. Am. Acad. Dermatol.* **12**, 497–506.
- Osborne, R., and Greenlee, W. F. (1985). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) enhances terminal differentiation of cultured human epidermal cells. *Toxicol. Appl. Pharmacol.* **77**, 434–443.
- Panteleyev, A. A., Paus, R., Ahmad, W., Sundberg, J. P., and Christiano, A. M. (1998). Molecular and functional aspects of the hairless (hr) gene in laboratory rodents and humans. *Exp. Dermatol.* **7**, 249–267.
- Panteleyev, A. A., Thiel, R., Wanner, R., Zhang, J., Roumak, V. S., Paus, R., Neubert, D., Henz, B. M., and Rosenbach, T. (1997). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) affects keratin 1 and keratin 17 gene expression and differentially induces keratinization in hairless mouse skin. *J. Invest. Dermatol.* **108**, 330–335.
- Parenteau, N. L., Bilbo, P., Nolte, C. J., Mason, V. S., and Rosenberg, M. (1992). The organotypic culture of human skin keratinocytes and fibroblasts to achieve form and function. *Cytotechnology* **9**, 163–171.
- Poland, A., and Glover, E. (1980). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin: Segregation of toxicity with the Ah locus. *Mol. Pharmacol.* **17**, 86–94.
- Poland, A., and Knutson, J. C. (1982). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. *Annu. Rev. Pharmacol. Toxicol.* **22**, 517–554.
- Prough, R. A. (1996). Introduction: Basal and inducible expression of cytochromes P450 and related enzymes. *FASEB J.* **10**, 807–808.
- Raskin, C. A. (1997). Apoptosis and cutaneous biology. *J. Am. Acad. Dermatol.* **36**, 885–896; quiz 897–888.
- Sadek, C. M., and Allen-Hoffmann, B. L. (1994). Cytochrome P450IA1 is rapidly induced in normal human keratinocytes in the absence of xenobiotics. *J. Biol. Chem.* **269**, 16067–16074.
- Safe, S. (1990). Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: Environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *Crit. Rev. Toxicol.* **21**, 51–88.
- Schmidt, J. V., and Bradfield, C. A. (1996). Ah receptor signaling pathways. *Annu. Rev. Cell Dev. Biol.* **12**, 55–89.
- Shebani, N., and Allen-Hoffman, B. L. (1994). Production of recombinant human involucrin using the glutathione-*S*-transferase system. In *Keratinocyte Methods* (I. M. Leigh and F. M. Watt, Eds.), pp. 167–174. Cambridge University Press, Cambridge.
- Simon, M. (1994). The epidermal cornified envelope and its precursors. In *The Keratinocyte Handbook* (I. Leigh, B. Lane, and F. Watt, Eds.), pp. 275–292. Cambridge University Press, Cambridge.
- Smola, H., Thiekotter, G., and Fusenig, N. E. (1993). Mutual induction of growth factor gene expression by epidermal–dermal cell interaction. *J. Cell Biol.* **122**, 417–429.
- Steinert, P. M., and Marekov, L. N. (1995). The proteins elafin, filaggrin, keratin intermediate filaments, loricrin, and small proline-rich proteins 1 and 2 are isodi-peptide cross-linked components of the human epidermal cornified cell envelope. *J. Biol. Chem.* **270**, 17702–17711.
- Sutter, T. R., Guzman, K., Dold, K. M., and Greenlee, W. F. (1991). Targets for dioxin: Genes for plasminogen activator inhibitor-2 and interleukin 1 β . *Science* **254**, 415–418.
- Van den Berg, M., Birnbaum, L., Bosveld, A. T. C., Brunstrom, B., Cook, P., Feeley, M., Giesy, J. P., Hanberg, A., Hasegawa, R., Kennedy, S. W., Kubiak, T., Larsen, J. C., van Leeuwen, F. X., Liem, A. K., Nolt, C., Peterson, R. E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M., Younes, M., Waern, F., and Zacharewski, T. (1998). Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ. Health Perspect.* **106**, 775–792.
- Weil, M., Raff, M. C., and Braga, V. M. (1999). Caspase activation in the terminal differentiation of human epidermal keratinocytes. *Curr. Biol.* **9**, 361–364.
- Wilk, R., Weizman, I., and Shilo, B. Z. (1996). Tracheless encodes a bHLH-PAS protein that is an inducer of tracheal cell fates in *Drosophila*. *Genes Dev.* **10**, 93–102.