Treatment of Normal Human Keratinocytes with 2,3,7,8-Tetrachlorodibenzo-p-dioxin Causes a Reduction in Cell Number, but No Increase in Apoptosis

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2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a persistent environmental toxin that has been studied extensively because of its deleterious effects on human and wildlife health. 2,3,7,8-Tetrachlorodibenzo-p-dioxin is the most potent congener of a family of halogenated aromatic hydrocarbons known as dioxins (reviewed in Safe, 1990; Van den Berg et al., 1998). TCDD causes a spectrum of pathologies in humans and rodents, including dermatopathology, thymic involution, and wasting (reviewed in Schmidt and Bradfield, 1996). Although the cellular and molecular basis of these pathologies is unknown, TCDD has been shown to cause apoptosis in a variety of cell types, including mammalian luteinized granulosa cells (Heimler et al., 1998) and Xenopus hepatocytes and erythrocytes (Sakamoto et al., 1995, 1997). Furthermore, evidence strongly suggests that a classical TCDD-induced pathology, thymic involution, is the direct result of precocious induction of apoptosis in thymocytes (Kamath et al., 1997; McConkey et al., 1988; Silverstone et al., 1994).

Skin, a stratified squamous epithelial tissue, functions as the body’s barrier against harmful environmental agents. Skin contains two layers, the dermis and the epidermis. The dermis, which is composed primarily of dermal fibroblasts and extracellular matrix proteins, provides the overlying epidermis with physical support, blood supply, and growth factor-mediated regulatory signals. The primary cellular component of the epidermis is the keratinocyte. Keratinocytes within the epidermis are organized into four layers based on morphological and biochemical properties: the basal, spinous, granular, and cornified layers (reviewed in Eckert et al., 1997). Keratinocytes in the basal layer, located most proximal to the dermis, actively proliferate and act as the source of all suprabasal keratinocytes within the epidermis. As basal cells divide, certain daughter cells lose contact with the basement membrane and begin to migrate 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 signals that trigger and control keratinocyte differentiation are largely unknown. Recently, it has been suggested that the cell biological characteristics of squamous differentiation are similar in many ways to those observed during apoptosis, a gene-regulated process of cell death (Gandarillas et al., 1999; Mitra et al., 1997; Weil et al., 1999). In both terminal differentiation and apoptosis, cells experience organelle breakdown and eventual enucleation. However, whereas apoptotic cells are fully degraded and engulfed by neighboring cells, differentiated keratinocytes remain present in the tissue.

In the studies presented here, we report a decrease in human keratinocyte cell number upon treatment of monolayer cultures with TCDD. Analysis of nuclear morphology, nucleosomal fragmentation, and caspase-3 activity revealed no increase in characteristics of apoptosis in TCDD-treated keratinocytes. TCDD-induced dermatopathologies, including epidermal hyperkeratosis and alterations in follicular morphology, have
been described in both humans and rodents. These effects are indicative of a perturbation in the regulatory processes that govern skin homeostasis. In order to investigate the cellular and molecular basis of TCDD-induced human dermatopathology, extensive in vitro studies have been performed using human keratinocytes in monolayer culture. However, results of these studies have been equivocal. For example, some groups report an increase in terminal differentiation (Greenlee et al., 1985; Osborne and Greenlee, 1985; Van Pelt et al., 1992), whereas others report increases in proliferation (Milstone and LaVigne, 1984). Furthermore, many of the investigations have employed squamous cell carcinoma lines instead of primary human keratinocytes (Gaido et al., 1992; Hudson et al., 1985). Finally, despite evidence indicating that TCDD induces apoptosis in some cell types, no analysis of TCDD-induced apoptosis in primary human keratinocytes has been performed to date. In our studies using primary human keratinocytes, we report a decrease in cell number in response to TCDD, which cannot be explained by an increase in apoptosis. We therefore conclude that TCDD alters an aspect of human keratinocyte homeostasis independent of apoptosis.

**MATERIALS AND METHODS**

**Monolayer cell culture.** Normal human keratinocytes were isolated from newborn human foreskin. Keratinocyte cultures were established by plating aliquots of a single-cell suspension in the presence of mitomycin C-treated Swiss mouse 3T3 M1 fibroblast feeder layer as previously described (Allen-Hoffmann and Rheinwald, 1984). The standard keratinocyte culture medium was composed of a mixture of Ham’s F-12 medium: Dulbecco’s modified Eagle’s medium (3:1, final calcium concentration 0.66 mM) supplemented with 2.5% fetal calf serum, 0.4 μg/ml hydrocortisone, 8.4 ng/ml cholera toxin, 5 μg/ml insulin, 24 μg/ml adenine, 10 ng/ml epidermal growth factor (EGF), and 100 units penicillin and 100 μg/ml streptomycin. Recombinant human EGF was obtained from R & D Systems (Minneapolis, MN). Prior to treatments, feeder layers were removed from keratinocytes with 0.02% EDTA.

**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 10⁻⁷ M. Solvent controls included 0.1% DMSO. Cultures were treated for 24 h unless otherwise noted.

**Growth studies.** When cultures of normal human keratinocytes were approximately 80% confluent, 3T3 feeder layers were selectively removed with 0.02% EDTA and keratinocytes were removed by trypsinization. For low calcium experiments, keratinocytes were seeded without a 3T3 feeder layer at 3 × 10⁵ cells per 60-mm dish in 0.05 mM calcium growth medium without EGF. For standard calcium experiments, keratinocytes were seeded without a feeder layer at 4 × 10⁵ cells per 60-mm dish in 0.66 mM calcium growth medium without EGF. After 24 h, cells were given fresh EGF-containing media at the appropriate calcium concentration. At this point, cells were treated with either TCDD or vehicle control. At each time point indicated, three dishes were trypsinized and the remaining plates were provided with fresh treatment medium.

**Evaluation of nuclear morphology.** For evaluation of nuclear morphology after 24 h exposure to TCDD, keratinocytes were plated in standard calcium (0.66 mM) medium onto glass coverslips in 35-mm dishes and allowed to grow to approximately 70% confluence. Cells were treated with either TCDD or vehicle control for 24 h. For the time-course experiment, cells were plated on coverslips in the amount and at the times outlined above for the standard calcium growth experiment. At the designated time of harvest, treatment medium was removed and samples were fixed overnight in 4% paraformaldehyde, rinsed with phosphate buffered saline pH 7.2 (PBS), stained with Hoechst 33258, and mounted with Vectashield. Slides were viewed with an IX-70 inverted fluorescence microscope (Olympus) equipped with a Hoechst (525 nm ± 20 nm) band-pass filter. At least 650 cells per slide were counted and those displaying apoptotic morphology were scored.

**RESULTS**

**TCDD Treatment of Keratinocytes Causes a Decrease in Cell Number**

Many of the reported TCDD-induced pathologies appear to stem from a disruption in normal tissue homeostasis. For example, chloracne, a TCDD-induced human dermatopathology, is characterized by epidermal hyperplasia and changes in hair follicle morphology. In an attempt to understand the specific mechanism by which TCDD exerts its effects on skin, we performed a series of experiments in which cultured human keratinocytes were exposed to TCDD.

Primary cultures of keratinocytes obtained from neonatal foreskins were seeded directly onto a plastic culture dish in either standard calcium (0.66 mM) or low calcium (0.05 mM) keratinocyte culturing medium without EGF. At standard calcium levels, cells are able to establish adhesive junctions with adjacent keratinocytes, whereas low calcium medium prevents formation of cell–cell contacts. After 24 h, cells were given either standard or low calcium keratinocyte medium containing EGF. At this point, cells were treated with either 10 nM TCDD or DMSO vehicle control. At 48-h intervals, three replicates each of treatment and control dishes from both calcium groups were trypsinized and cells were counted. Over an 8- or 10-day
period, we observed a decrease in cell number in TCDD-treated cultures compared to controls, regardless of the calcium concentration of growth media (Fig. 1).

**TCDD Does Not Cause Apoptosis in Keratinocytes in Monolayer Culture**

We next investigated whether the decrease in cell number observed in TCDD-treated cultures was due to an increase in apoptosis. In order to determine whether TCDD-treated keratinocytes were undergoing apoptosis, we assessed three different endpoints: nucleosomal fragmentation, morphological changes, and caspase-3 activity. We chose this combination of assays because they assess both biochemical and cell biological changes and span a temporal range in the apoptotic process. The growth and terminal differentiation characteristics of keratinocytes grown in vitro are tightly linked to their level of confluence in culture. In order to ensure consistency, all assays were performed on cultures that were 70–80% confluent. Furthermore, observation of cultures prior to execution of the assays revealed small, basal-like cells, indicative of undifferentiated keratinocytes (Watt and Green, 1981).

A relatively late-stage endpoint of apoptosis is nuclear condensation and blebbing (Loo, 1998). Morphological analysis of Hoechst-stained control keratinocytes revealed nuclear condensation, blebbing, or apoptotic bodies in less than 1% of cells analyzed. TCDD treatment for 24 h did not alter the number of nuclei displaying the morphological indicators of apoptosis (Fig. 2). Keratinocytes exposed to UVB or 5 μg/ml cycloheximide exhibited massive condensation and blebbing 24 h after exposure (data not shown). A time-course experiment in which keratinocytes were grown in the presence of TCDD for up to 10 days showed no induction of the morphological characteristics of apoptosis even after prolonged exposure to TCDD (Fig. 3).

Nucleosomal fragmentation is another hallmark of apoptosis (Loo, 1998). As a result of activation of a cascade of proteolytic caspase enzymes, cellular structures and DNA are broken down in an orderly fashion. Cells exposed to an apoptotic

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**Fig. 1.** TCDD treatment of keratinocytes causes a decrease in cell number. Primary human keratinocytes were grown without a feeder layer in (A) 0.66 mM Ca²⁺ media or (B) 0.05 mM Ca²⁺ media and treated with either 10 nM TCDD (○) or DMSO (●). At each time point indicated, three plates for each treatment were harvested and adherent cells were counted.

**Fig. 2.** TCDD does not induce the morphological changes characteristic of apoptosis in keratinocytes in monolayer culture. Keratinocytes were grown on glass coverslips and treated for 24 h with TCDD or DMSO, fixed, and stained with Hoechst. At least 650 cells were counted per coverslip and three coverslips per treatment were counted for each experiment. Three different experiments are shown with standard error.
stimulus can be lysed and a “ladder” of regularly spaced DNA fragments can be detected. Nucleosomal fragmentation is considered a relatively late-stage event in the apoptotic process. Cells used for analysis of nucleosomal fragmentation were treated for 24 h with either TCDD or vehicle control and were lysed shortly before achieving confluence. TCDD-treated keratinocytes revealed a very faint laddering pattern, which was no more intense than untreated or vehicle-treated cells (Fig. 4). As a positive control, keratinocytes were suspended in semisolid medium, a procedure known to induce nucleosomal fragmentation in keratinocytes (Sachsenmeier et al., 1996). As expected, an intense laddering pattern was observed in the positive control samples. We therefore conclude that TCDD does not induce nucleosomal fragmentation in keratinocytes in monolayer culture.

Finally, we measured caspase-3 activity in TCDD and control keratinocytes. Caspase-3 is considered the first effector caspase in the autoregulatory cascade of caspase proteases that become activated during apoptosis (reviewed in Hengartner, 2000). Caspase-3 activity can be measured by exposing cell lysates to a fluorescently tagged tetrapeptide, DEVD-AMC, which is a specific substrate for caspase-3. In our experiments, keratinocytes were exposed to either TCDD or vehicle control for 24 h. Cells were then lysed, exposed to the fluorogenic tetrapeptide, and the resulting fluorescence was measured. We observed no consistent difference in caspase-3 activity in TCDD-treated samples compared to vehicle controls (Fig. 5). Fluorescence measured in the positive control sample, UVB-exposed keratinocytes, was three to five times greater than that observed in any of the experimental samples. The substantial increase in caspase-3 activity observed in UVB-exposed keratinocytes was measured 7 h after exposure, indicating that

FIG. 3. Extended exposure to TCDD does not induce the morphological changes characteristic of apoptosis in keratinocytes in monolayer culture. Keratinocytes were grown on glass coverslips and treated for up to 10 days with TCDD (○) or DMSO (●). At each time point indicated, three coverslips for each treatment were fixed and stained with Hoechst. At least 700 cells per coverslip were counted. Results are shown with standard error bars (in some cases error is too small for bar to be visible).

FIG. 4. TCDD does not cause an increase in DNA laddering in keratinocytes in monolayer culture. Cell lysates were collected from adherent, untreated (A), suspended (S), DMSO-treated (D), and TCDD-treated (T) keratinocytes. A molecular weight marker was also loaded (L). DNA was isolated, 3’ end-labeled with [32P]ddATP, displayed on a 1.5% agarose gel, and subjected to autoradiography. Data from two different experiments are shown.

FIG. 5. TCDD does not cause an increase in caspase-3 activity in keratinocytes in monolayer culture. Keratinocytes were treated with either DMSO or TCDD for 24 h. Cell lysates were collected and assayed for caspase-3 activity in triplicate. Data from three different experiments are shown with standard error, using bars of a different color or pattern for each experiment.
keratinocytes are able to rapidly activate an apoptotic response. In summary, at least three sets of experiments per apoptosis assay were performed, all of which showed no differences in amount of apoptosis between TCDD- and vehicle-treated samples.

**DISCUSSION**

Our data indicate that TCDD causes a reduction in keratinocyte cell number in monolayer culture and that the reduction is not the result of increased apoptosis. Upon initial observation that treatment of keratinocytes with TCDD caused a reduction in cell number, we reasoned that TCDD might be inducing apoptosis in keratinocytes in a manner similar to that observed in other cell types. However, our data have shown that none of the characteristics typically associated with apoptosis are triggered by TCDD treatment of normal human keratinocytes, leading to two important conclusions. First, TCDD disrupts keratinocyte homeostasis without increasing the incidence of cell death. Second, the ability of TCDD to induce apoptosis is a cell type-specific phenomenon.

Several observations have established a link between TCDD and apoptosis. TCDD has been shown to cause apoptosis in a variety of mammalian and *Xenopus* cell types (Heimler et al., 1998; Kamath et al., 1997; McConkey et al., 1988; Sakamoto et al., 1995, 1997). The ability of TCDD to initiate precocious apoptosis in thymocytes is particularly interesting because it provides a mechanistic explanation for the thymic involution observed in TCDD-exposed animals. Animal studies have shown that TCDD induces apoptosis in thymocytes in vivo (Kamath et al., 1997). Furthermore, studies using Fas and Fas ligand (FasL) null animals have shown that these molecules, which are known to be involved in the initiation of apoptosis, are also involved in the induction by TCDD of apoptosis in thymocytes (Kamath et al., 1999). Similar to thymocytes, at least some forms of keratinocyte apoptosis are dependent on FasL. For example, UV exposure of FasL-deficient mice reveals an inability of these animals to eliminate damaged cells via apoptotic pathways (Hill et al., 1999). The similarity between thymocyte response to TCDD and keratinocyte response to UVB led us to hypothesize that keratinocytes could also respond to TCDD by activating the apoptotic cascade. Our findings do not support this hypothesis.

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). For example, catagen, regression of the hair follicle, occurs regularly during normal hair cycling in healthy skin (reviewed in Paus, 1998). Keratinocytes that have been damaged by UVB radiation or that have begun to undergo cancerous transformation also initiate apoptotic pathways (Benassi et al., 1997; Evan and Littlewood, 1998). Furthermore, excessive apoptosis has been reported in noncancerous disease states such as psoriasis (reviewed in Karasek, 1999). These observations strongly suggest 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 (Takahashi et al., 1998; Weil et al., 1999).

The reduction in keratinocyte number we have observed is indicative of a change in the homeostatic balance of TCDD-treated cultures compared to untreated controls. This imbalance could be caused by any one of several cellular changes, including increased terminal differentiation, decreased proliferation, or increased apoptosis. Although TCDD has been shown to induce apoptosis in some cell types, our data indicates that this is not the case in keratinocytes.

Confluence of keratinocyte cultures at the time of treatment could influence results observed in apoptosis assays. Milstone and LaVigne (1984) observed increased keratinocyte proliferation in response to TCDD only in confluent cultures. These authors argue that, like intact skin, confluent keratinocyte cultures maintain a dynamic balance between proliferation and terminal differentiation. They therefore propose that such cultures provide a unique system in which to analyze the pharmacological action of agents on skin (Milstone, 1983). Although the ability of TCDD to alter proliferative characteristics of keratinocytes may depend on confluence, it is unlikely that treatment of confluent keratinocyte cultures with TCDD would have produced any different results than we observed in preconfluent cultures. Chaturvedi et al. (1999) examined the effect of confluence on the ability of UV irradiation to induce apoptosis in primary human keratinocytes. They found a lower percentage of TUNEL-positive cells in confluent cultures than in preconfluent cultures. Furthermore, preliminary assays for detection of nucleosomal fragmentation and caspase-3 activity performed as part of our study revealed no TCDD-induced increase in apoptosis in confluent cultures. Finally, exposure of keratinocytes in organotypic culture to TCDD shows no increase in apoptosis (Loertscher et al., 2001). Keratinocytes grown in organotypic culture exist in a microenvironment that could be considered confluent.

In addition to affecting the apoptotic cascade in some cell types, the molecular pathway activated by TCDD is also involved in cell cycle regulation. The aryl receptor (AhR), the protein which is thought to mediate TCDD toxicity, has been shown to be involved in cell cycle arrest. Ma and Whitlock (1996) reported that AhR-deficient hepatoma cells have a longer doubling time than cells with the normal amount of AhR. This defect can be corrected by stable transfection of deficient cells with AhR. Subsequently, two different groups independently illustrated that AhR physically interacts with the retinoblastoma protein (RB) (Ge and Elferink, 1998; Puga et al., 2000). RB is thought to induce cell cycle arrest through transcriptional repression of E2F. Puga and coworkers (2000) showed that AhR, although unable to repress E2F transcription
on its own, potentiates the action of RB, resulting in cell cycle arrest. The authors therefore speculate that AhR acts as an environmental sensor that modulates cellular activity in response to environmental stimuli. If AhR does indeed function to sense environmental insult and subsequently to initiate a cell type-specific response, the authors argue that this could explain the spectrum of effects observed in different cell types in response to TCDD. That is to say that AhR may aid each individual cell type to respond to environmental insult in a way that would minimize ultimate toxicity to a whole organism. In the case of keratinocytes, cell cycle arrest in response to TCDD seems unlikely, since several published studies report an increase in expression of the mitogenic protein transforming growth factor-α upon treatment of keratinocytes with TCDD (Choi et al., 1991; Hudson et al., 1986). However, it is plausible that TCDD may exert its effects on keratinocytes by altering the normal terminal differentiation program. Accelerating terminal differentiation in skin could conceivably allow for more rapid clearance of a toxin through increased shedding of keratinocytes. This assertion is supported by the observation that treatment of human keratinocytes and fibroblasts in a three-dimensional, organotypic culturing system results in alterations in the differentiation characteristics of the cultures with no changes in proliferation or apoptosis (Loertscher et al., 2001).

In summary, we have shown that TCDD causes a decrease in keratinocyte cell number without inducing apoptosis. These findings indicate that the effect of TCDD is cell-type specific and involves disruption of an aspect of keratinocyte homeostasis that cannot be simply explained by the activation of a cell death pathway. An understanding of the effects of TCDD at the molecular level, coupled with a more complete human skin model, will be necessary to fully understand the basis of TCDD dermatotoxicity.

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