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Toxicity of Chlorinated Aromatic Compounds in Animals and Humans: *In Vitro* Approaches to Toxic Mechanisms and Risk Assessment

by William F. Greenlee,* Rosemarie Osborne,* Karen M. Dold,* Laurie G. Hudson† and William A. Toscano, Jr.†

Human exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and chlorinated analogs commonly results in pathological changes in the skin and its appendages characterized by thickening of the epidermis (acanthosis), hyperkeratosis and squamous metaplasia of the epithelial lining of the sebaceous glands. Acneform lesions (chloracne) develop as hair follicles dilate and fill with keratin and sebaceous glands become cystic. In animal models it has been found that the chloracneogenic potential of the halogenated aromatic compounds examined corresponds with the relative affinity of these same compounds for the cytosolic TCDD receptor. This receptor controls the coordinate expression of a number of inducible enzyme activities and in certain cell targets can alter normal programs of proliferation and differentiation. In this report we describe some of our ongoing studies on the mechanisms of action of TCDD in normal human epidermal cells and squamous cell carcinoma (SCC) lines. These systems permit detailed investigation of the molecular and biochemical events underlying pathologic changes in the skin and offer the potential of establishing a risk assessment model for halogenated aromatic compounds by using human target cells.

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the prototype for the halogenated aromatic compounds including the polychlorinated biphenyls (PCBs) (*1,2*).‡ Studies in animals indicate that TCDD and isosteric analogs act through a common receptor to produce characteristic patterns of toxic and biochemical responses (*2,3*). Toxic responses commonly observed in most animal species include a slow wasting syndrome, teratogenesis, and thymic atrophy (*4–6*). Species-specific responses include hepatoxicity, edema of the pericardium, hyperkeratosis and chloracne, and at high doses, acute lethality (*1,2,7–9*).

A number of clinical abnormalities have been reported in individuals exposed to various halogenated aromatic compounds. Chloracne is one of the most sensitive and widespread responses observed in humans (*10,11*). Other symptoms include weight loss, impaired liver function, hepatic porphyria, general malaise, and peripheral neuropathies (*12*). These are discussed in greater detail elsewhere in this volume (see section on clinical aspects of human toxicity). This report focuses on model cell culture systems for mechanistic studies on the toxicity of chlorinated aromatic compounds to the human epidermis and the potential value of these systems in risk assessment.

TCDD is the most potent of the halogenated aromatic compounds, a property presumably associated with its relative inertness to biological and chemical degradation (*13*) and its high affinity for the cytosolic receptor (*14*). Other members of this class which are more readily metabolized, such as the PCBs, possess similar affinities for the cytosolic receptor measured *in vitro* (*15*), but vary widely in their toxic potency (*1,3*), indicating the importance of considering both pharmacokinetic and mechanistic studies in estimating human health risks.

Ah Locus: Role in Mediating Toxicity of Chlorinated Aromatic Compounds

In certain inbred murine strains, the induction by TCDD of cytochrome(s) P_1-450 and several associated

*Department of Cell Biology, Chemical Industry Institute of Toxicology, P.O. Box 12137, Research Triangle Park, NC 27709
†Charles A. Dana Laboratory of Toxicology, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115

‡The term "halogenated aromatic compounds" is commonly used to denote a large series of compounds isosteric with TCDD. These include halogenated dibenzo-*p*-dioxins, dibenzofurans, biphenyls, biphenylenes, and azo- and azoxybenzenes.

monooxygenase activities, including aryl hydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin O-deethylase (ECOD), is regulated by a single genetic locus (designated the *Ah* locus) and its putative gene product, the TCDD receptor protein (*14,16,17*). In these same murine strains, TCDD-induced thymic atrophy and cleft palate formation segregate with the *Ah* locus (*18*). Recent investigations have also shown that TCDD produces epidermal hyperplasia (*19*) and promotes skin papillomas (*20*) in hairless mice bearing the recessive mutation *hr/hr*. Further, the data indicate that TCDD-induced epidermal hyperplasia requires interaction between two regulatory gene loci, *Ah* and *hr* (*19*). Based on these and other findings (*2,18–20*), it has been postulated that the murine *Ah* locus, either singly or in concert with other regulatory genes, controls at least two distinct pleiotropic responses: a limited, but widely expressed gene battery which includes the structural genes for cytochrome(s) P_1-450, and in a few organs such as skin and thymus, a second gene battery regulating cell proliferation and differentiation (*3*).

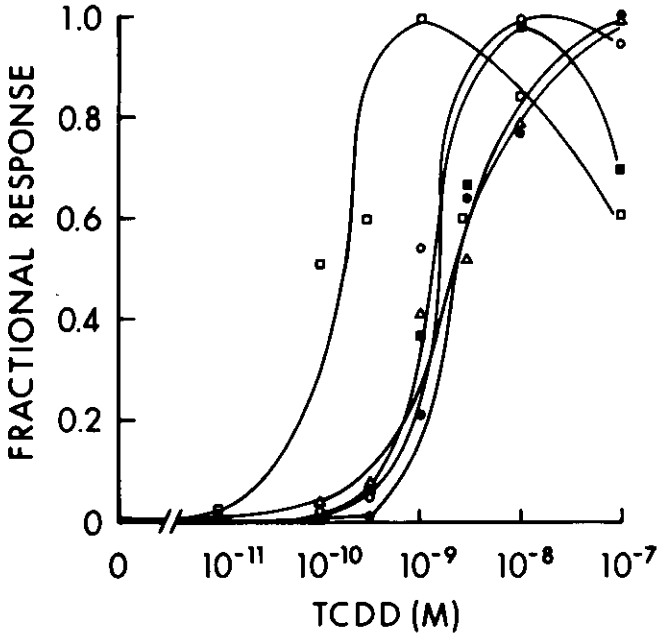

FIGURE 1. Fractional log dose–response curves for the induction of ECOD activity in human SCC cell lines by TCDD: (○) SCC-9; (●) SCC-13; (□) SCC-15; (■) SCC-12F; (Δ) SCC-4. SCC cells were seeded at a density of 10^4 cells /60 mm plastic culture dish in 4 mL of growth medium (Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 100 μg/mL each of penicillin and streptomycin; this is the growth medium used in all the experiments with SCC cells described in this report) in the presence of a feeder layer of 10 lethally irradiated murine 3T3 fibroblasts. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2:95% air, and the growth medium was changed every third day. At confluence, growth medium containing the indicated concentration of TCDD was added. After 2 days the cultures were washed three times with 0.02% EDTA in phosphate-buffered saline and ECOD activity was assayed as described previously (*25,41*). Fractional responses were calculated by equating maximally induced activity to 1.0 and control activity to 0. Taken from Hudson et al. (*25*), reproduced with permission of the publisher.

Evidence for the *Ah* Locus in Human Cells

The presence of the *Ah* locus in human cells, suggested by findings on the induction of AHH activity in cultured mitogen-activated lymphocytes (*21*), has not been conclusively established. Studies on the induction of AHH activity in cultured lymphocytes (*22*) and monocytes (*23*) from monozygotic and dizygotic twins confirm a heritable component, but are not able to distinguish between a monogenic or polygenic mode of inheritance (*22*). Chromosome mapping in mouse–human cell hybrids suggests that either the structural or the regulatory genes for the induction of AHH activity are located on human chromosome 2; however, participation of mouse genes in the observed response is not ruled out (*24*).

We examined the responsiveness of cultured human squamous cell carcinoma (SCC) lines derived from tumors of the epidermis and tongue to TCDD by measuring the induction of ECOD activity (Fig. 1) (*25*). In four of the SCC lines, the EC_{50} (concentration required to elicit 50% of the maximal response) is approximately 10^{-9} M, whereas in one line the EC_{50} is 10^{-10} M. In each of the less sensitive lines a concentration of 10^{-10} M TCDD elicits less than 5% of the maximal enzyme activity. Specific binding of radiolabeled TCDD is detected in the cytosol fraction from all the SCC lines (Table 1) and the relative amount of receptor in each line correlates with maximally induced ECOD activity (*25*). These data indicate that human cell lines derived from a target tissue for TCDD toxicity contain the TCDD receptor and show differential sensitivity to TCDD

Table 1. Specific binding of [^{3}H]TCDD to cytosol fractions from SCC lines.

Cell line	Specific binding, fmole/mg cytosol protein[a]	Relative response: Specific binding[b]	Relative response: ECOD activity[c]
SCC-9	9.2 (0.6)	5.3	5.2
SCC-15	6.1 (1.8)	3.5	2.2
SCC-12F	1.8 (0.3)	1.0	1.0

[a]Specific binding was measured by sucrose-density gradient analysis as described previously (*25*). Values shown represent the average from two experiments. The range is given in parentheses. The total amount of protein added to the gradient ranged between 4 to 5 mg and the ratio of total to nonspecific binding was approximately 5 to 1. Single determinations on lines SCC-13 and SCC-4 gave values of 8.5 and 5.8 fmole/mg cytosol protein, respectively. Taken from Hudson et al. (*25*).

[b]Calculated from the average values for the specific binding in each line and normalized with respect to the value obtained for line SCC-12F.

[c]Determined from the maximally induced activities given in Fig. 3 and normalized with respect to the value obtained for line SCC-12F.

analogous to the murine strain differences in sensitivity regulated by the *Ah* locus (*25*).

Model Cell Culture Systems to Study Mechanisms of Epidermal Toxicity

The skin lesions produced by TCDD and halogenated isostereomers are typified by thickening of the epidermis (acanthosis), hyperkeratosis and squamous metaplasia of the epithelial lining of the sebaceous glands (*4,8*). Acneform lesions develop as hair follicles dilate and become filled with keratinaceous material and sebaceous glands become cystic (*12*). In animal models the available data on structure–activity relationships indicate that the chloracneogenic potential of halogenated aromatic compounds corresponds with the capacity of these same compounds to bind to the TCDD receptor and evoke biochemical and toxic responses in other tissues (*2,18,19,26,27*). It would appear that the same structure–activity relationship can be used to predict skin toxicity in humans (*2,28*).

Two epithelial keratinizing cell types have been successfully serially cultivated by using lethally irradiated 3T3 murine fibroblast feeder layers; XB cells, a cell line derived from a mouse teratoma (*29*); and normal human epidermal cells, usually obtained from neonatal foreskin (*30*). Monoclonal colonies consisting of multiple cell layers appear in culture. Within each colony, proliferating cells are confined to the basal layer. As cells migrate to upper layers, they lose their capacity to divide and begin the process of terminal differentiation. This is marked by an increase in cell size, changes in keratin expression, and the formation of crosslinked cornified envelopes (*31*). Flattened and elongated squames are shed into the media.

TCDD elicits a concentration-dependent keratinization response in cultured murine XB cells (*32*). It was suggested that the keratinization response in these cells was mediated by the TCDD receptor based on dose–response parameters and structure–activity relationship data comparing the potencies of halogenated aromatic compounds to induce keratinization in XB cells with their relative affinities for the TCDD receptor in murine hepatic cytosol (*32*).

We have found that TCDD enhances stratification (Fig. 2) and induces hyperkeratinization (as judged by the intensity of Rhodanile Blue staining, Fig. 3) in early passage human epidermal cells (*33*). These responses to TCDD are associated with a 30 to 40% inhibition of both basal and epidermal growth factor (EGF)-stimulated DNA synthesis (*33*). Similar results were observed in a human SCC line (SCC-12F) with growth requirements similar to normal epidermal cells (*33,34*). These findings are consistent with an enhanced commitment of proliferating basal cells to terminal differentiation and suggest that these *in vitro* systems are appropriate models for hyperkeratinization observed *in vivo* (*4,12*).

Regulation of Biochemical Mediators of Human Epidermal Cell Proliferation by TCDD

The proliferation and differentiation of epidermal cells are regulated by several biochemical mediators including hydrocortisone, cyclic nucleotides and EGF (*31*). In the previous section it was noted that TCDD enhances keratinization and inhibits EGF-stimulated DNA synthesis in SCC-12F cells. As shown in Figure 4, treatment of these cells with TCDD results in a concentration-dependent decrease in the specific binding of EGF to a value 40% of control with an EC of 1 nM. Scatchard analysis of EGF binding indicates that TCDD exposure results in a loss of high affinity ($K_d = 2.8 \times 10^{-10}$ M) EGF binding sites (*34,35*).

A structure–activity relationship for down regulation of EGF binding is shown in Table 2. The response of cells treated with 2,3,7,8-tetrachloro-dibenzofuran, an isostereomer of TCDD, is equal to that with TCDD, whereas no significant alteration in EGF binding occurs in cells treated with the nonisostereomeric analog, 2,7-dichlorodibenzo-*p*-dioxin. The observed stereospecificity (Table 2) and potency (EC_{50} = 1 nM, Fig. 4) suggest that the down regulation of EGF receptors in SCC-12F cells by TCDD is mediated by the TCDD receptor.

The kinetics of inhibition of EGF binding in SCC-12F cells by TCDD and benzo(a)pyrene differ (Table 3). Maximum inhibition induced by benzo(a)pyrene, an agent which inhibits EGF binding in C3H10T½ cells (*36*) and hepatoma cell lines (*37*), occurs within 24 hr. with 90% recovery by 48 hr. In contrast, TCDD requires 72 hr to produce maximum inhibition, and no recovery is observed 10 days after removal of TCDD from the culture medium (*34,35*). TCDD produces hyperkeratinization in SCC-12F cells (*33*) and is characterized by its ability to produce a prolonged decrease in EGF binding.

Table 2. Inhibition of EGF specific binding by TCDD and chlorinated analogs.

Treatment[a]	Specific binding, fmole/mg protein[b]	Binding, % of control
DMSO (0.1%)	8.92 ± 0.93	100
2,7-DpD (10^{-6} M)	8.41 ± 0.67	94
TCDBF (3×10^{-8} M)	2.97 ± 0.21	33
TCDD (10^{-8} M)	3.38 ± 0.52	38

[a]Confluent cultures of SCC-12F cells were treated with the indicated compounds or solvent vehicle for 72 hr prior to the measurement of EGF specific binding (*34,35*). TCDBF, 2,3,7,8-tetrachlorodibenzofuran; 2,7-DpD, 2,7-dichlorodibenzo-*p*-dioxin. Adapted from Hudson et al. (*34,35*).

[b]Values shown are the means (± SE) of determinations on triplicate cultures normalized with respect to total protein.

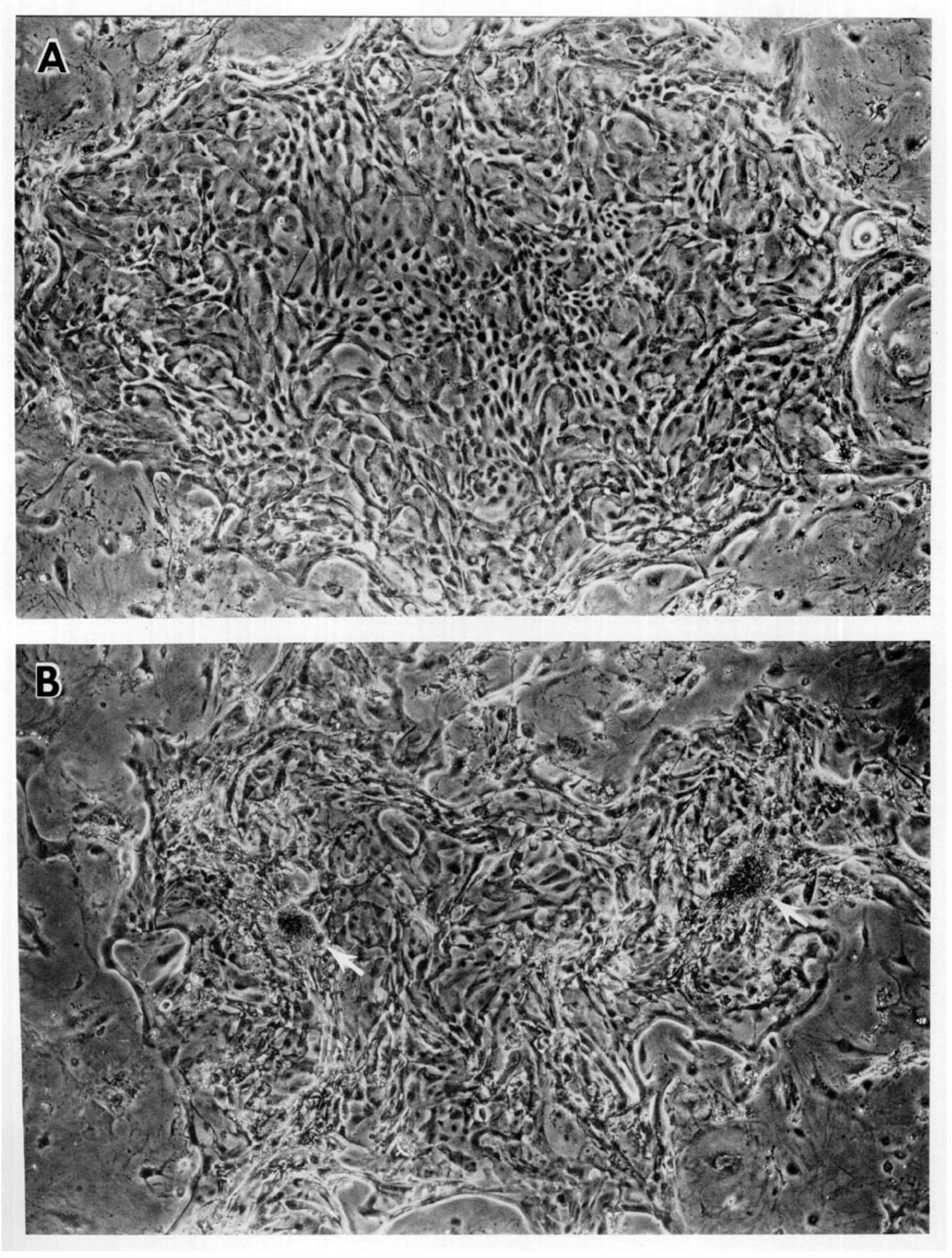

FIGURE 2. Enhanced stratification in colonies of normal human epidermal cells treated with TCDD: (*A*) Control, ×40; (*B*) TCDD, ×40, arrows indicate areas of enhanced stratification.

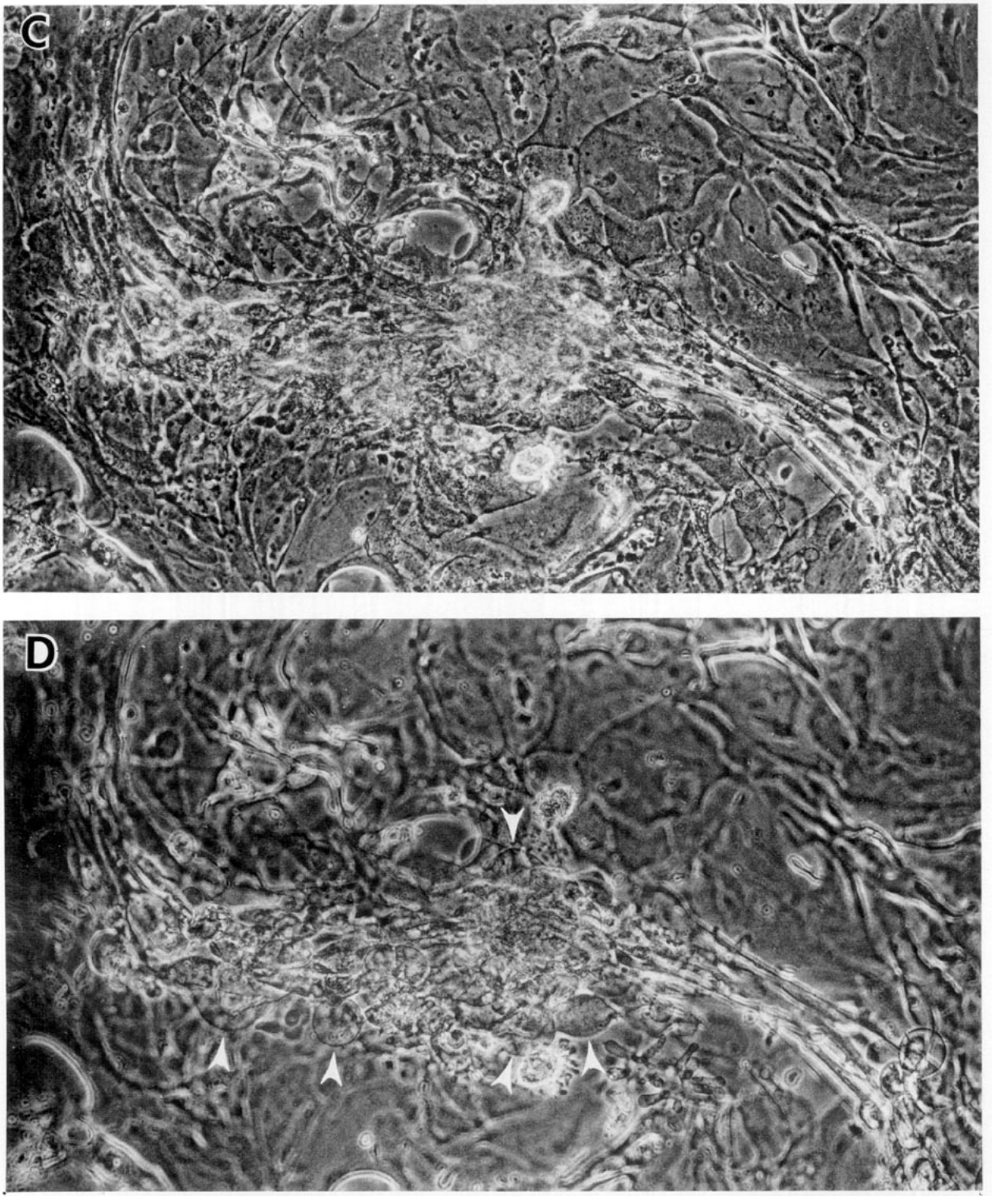

FIGURE 2 (cont'd). (*C*) TCDD, ×100; area shown is that indicated by the arrow to the left in *B*; (*D*) TCDD, ×100; same field as in *C* but focused on a more superficial cell layer. Note the loosely adherent squames, which lack discernible intracellular organelles, as indicated by the arrowheads. Cells were plated at a density of 5×10^3 cells/60 mm plastic culture dish and were grown as described in the legend to Fig. 1 except that the growth medium used for normal epidermal cells was 75% Dulbecco's modified Eagle's medium: 25% Ham's F12 medium supplemented with 5% fetal bovine serum, 10 ng/mL epidermal growth factor, 0.4 μg/mL hydrocortisone, 10 ng/mL cholera toxin, and 100 μg/mL each of penicillin and streptomycin. Two days later, growth medium containing TCDD (10 nM) or solvent vehicle (0.1% *p*-dioxane) was added. The medium was replaced every 3–4 days with fresh growth medium containing the appropriate additions and the cells were maintained in culture for 2 weeks. At the end of the treatment period, colonies of living cells were photographed under phase contrast.

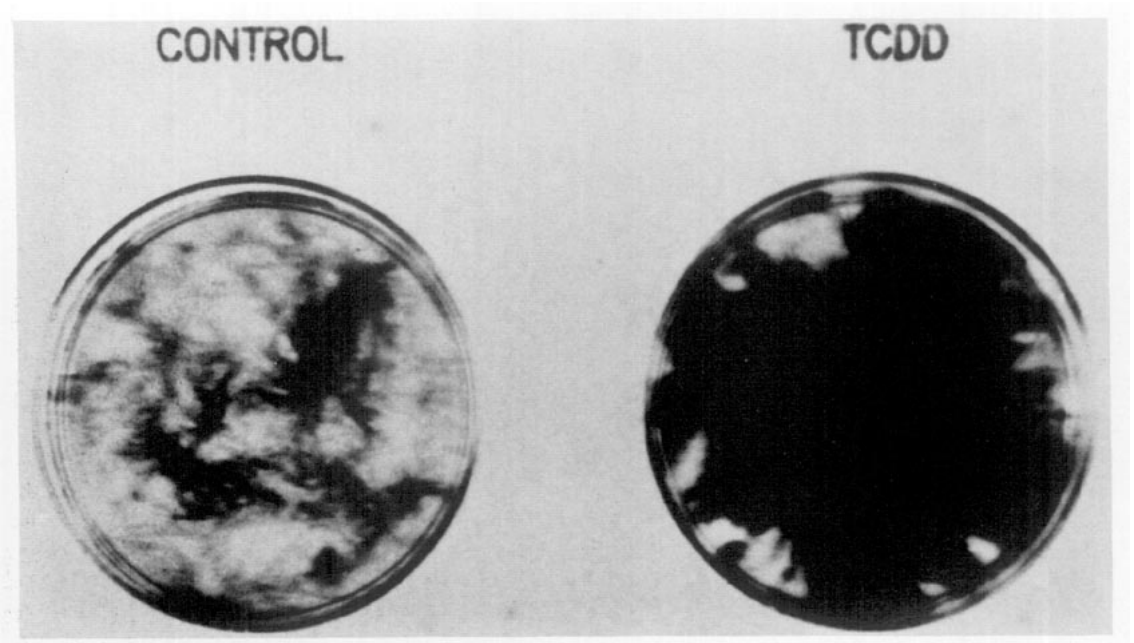

FIGURE 3. TCDD-induced hyperkeratinization in cultured normal human epidermal cells. Cells were plated at a density of 3×10^5 and were grown to confluence using the methods described in the legends to Figs. 1 and 2. The confluent cultures were treated with TCDD (10 nM) or solvent vehicle (0.1% *p*-dioxane) and maintained in culture for 2 weeks. At the end of the treatment period, the cultures were fixed and stained with Rhodanile Blue (*30*). The intensity of staining is a measure of the degree of keratinization.

Benzo(a)pyrene, which binds to the cytosolic TCDD receptor, but to our knowledge has not been reported to produce hyperkeratinization *in vivo*, produces a transient inhibition of EGF binding. These data comparing the actions of TCDD and benzo(a)pyrene suggest that a sustained refractoriness to growth factors such as EGF may be important in the expression of hyperkeratinization.

Several lines of evidence indicate that enhanced proliferation of human epidermal cells is associated with increased concentrations of intracellular cyclic AMP (cAMP) (*38*). In initial studies we have examined the actions of TCDD on the regulation of adenylate cyclase activity in human SCC lines with different growth responses to TCDD. Under appropriate culture conditions, treatment of line SCC-9 with TCDD enhances colony expansion (*39*), whereas in line SCC-12F, TCDD inhibits colony expansion (*39*). In SCC-9 cells TCDD appears to act through a unique membrane receptor mechanism to stimulate adenylate cyclase activity nearly 2-fold (*40*). The mechanisms for the modulation of adenylate cyclase activity by TCDD are not known; however, preliminary evidence suggests that TCDD may alter the responsiveness of the cyclase system to several well-characterized homone activators (M. J. Young, personal communication).

Summary and Conclusions

Treatment of early passage normal epidermal cells in culture with TCDD enhances stratification in cell colonies (Fig. 2), increases keratinization in confluent cultures (Fig. 3) and inhibits both basal and EGF-stimulated DNA synthesis (*33,34*). These results indicate enhanced cell differentiation in the presence of TCDD and suggest that TCDD stimulates the commitment of proliferating basal cells to a program of terminal differentiation.

Table 3. Kinetics of inhibition of EGF specific binding by TCDD and benzo(a)pyrene.

Treatment[a]	Time, hr	Specific binding, % of control
TCDD	12	90
(10^{-8} M)	24	80
	48	58
	72	40
Benzo(a)pyrene	12	70
(10^{-6} M)	24	60
	48	90

[a]Confluent cultures of SCC-12F cells were treated with solvent vehicle (DMSO, 0.1%), TCDD, or benzo(a)pyrene for the times indicated prior to the determination of EGF specific binding. Values shown are normalized with respect to total protein. Adapted from Hudson et al. (*34,35*).

Using prototype SCC lines which under certain culture conditions display enhanced proliferation (SCC-9) and differentiation (SCC-12F) in the presence of TCDD, studies were carried out to determine potential biochemical mechanisms for these responses. In SCC-12F cells, TCDD treatment results in a selective loss of high affinity EGF receptors (*34,35*) rendering these cells refractory to the growth stimulating effects of EGF. The dose–response parameters (Fig. 4) and stereospecificity (Table 2) of this response suggest that altered regula-

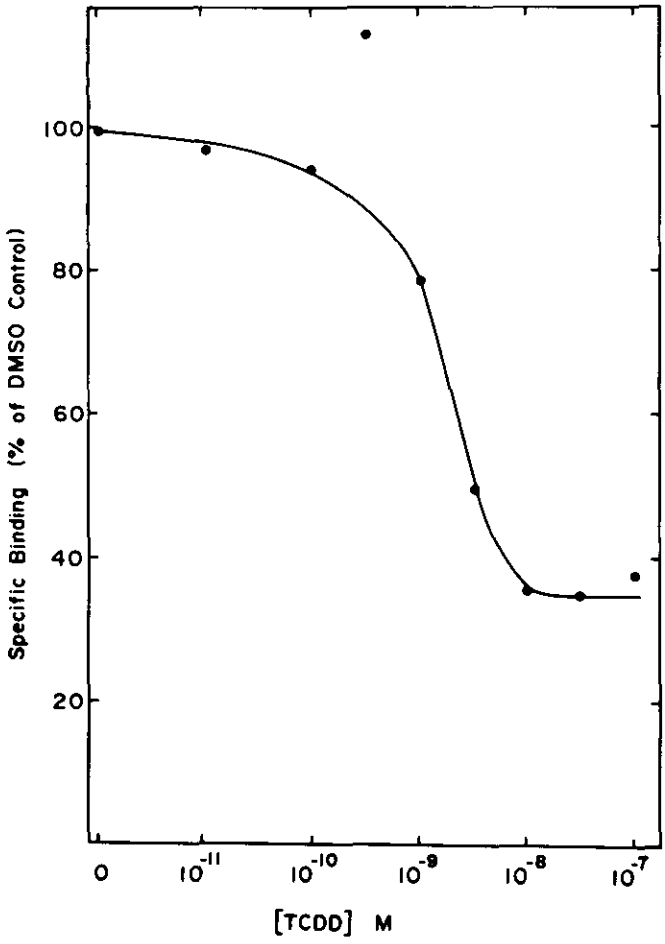

FIGURE 4. Concentration-dependent inhibition of EGF specific binding to SCC-12F cells by TCDD. Confluent cultures of SCC-12F cells were treated in triplicate with TCDD (10^{-11} to 10^{-7}M) for 72 hr prior to the determination of EGF specific binding. Specific binding is expressed as a percentage of the DMSO control and is normalized with respect to total protein. The absolute value for the DMSO control was 8.9 ± 0.6 fmole/mg protein. Taken from Hudson et al. (*34,35*).

tion of EGF binding by TCDD is mediated by the cytosolic receptor shown to be present in all of the SCC lines (Table 1). Under conditions favoring enhanced proliferation in SCC-9 cells, TCDD does not markedly inhibit EGF binding (L. G. Hudson, unpublished observations). TCDD appears to act through a unique receptor mechanism (*40*) to stimulate adenylate cyclase activity nearly 2-fold.

These data suggest that the hyperkeratinization and hyperplastic responses of epidermal cells to TCDD result from actions of TCDD at 2 (or more) membrane sites. Hyperkeratinization may result from the prolonged down regulation of receptors for growth factors such as EGF. The inability to respond to growth factors may then lead to enhanced commitment to terminal differentiation. Hyperplasia, a response commonly seen in the interfollicular epidermis (*12*), may result from a direct and specific activation of adenylate cyclase activity by TCDD increasing the intracellular concentration of cAMP, a positive mediator of epidermal cell proliferation (*38*). The contrast in the actions of TCDD in SCC-9 cells versus SCC-12F and normal epidermal cells, as described above, suggests that the response of a specific target cell to TCDD depends on the unique balance of regulatory mechanisms controlling cell division and differentiation in that cell.

Cultured Epidermal Cells As a Risk Assessment Model for Halogenated Aromatic Compounds

The results presented above demonstrate that normal human epidermal cells and human SCC lines are valuable *in vitro* models for dissecting the biochemical events responsible for actions of TCDD on the proliferation and differentiation of human target cells to TCDD. These systems should also prove useful for screening chloracneogens in quantitative risk assessment. Parameters such as cell growth rates, DNA synthesis, keratin expression, crosslinked envelope formation, EGF binding and stimulation of adenylate cyclase activity can be quantitated and are relevant to the spectrum of skin-associated toxicities resulting from exposure to TCDD. Further, since the skin is the first site of contact in many environmental exposure episodes, direct action of suspect agents on the cultured cells would appear to be relevant. However, in attempting to quantitate risk it is also important to consider the combined ability of the liver and the skin to metabolize and inactivate halogenated compounds. For example, metabolic inactivation of compounds such as the PCBs may greatly reduce the potential risk for chloracne relative to metabolically stable compounds such as TCDD. Cytochrome(s) P_1-450-associated enzyme activities are inducible in all of the human SCC lines examined (Fig. 1) and in normal human epidermal cells (W. F. Greenlee, unpublished observations). Studies are under way to characterize the biotransformation potential of cultured epidermal cells. The differences in sensitivity for induction of ECOD activity by TCDD in SCC lines (Fig. 1) and differential growth responses to TCDD (*25,33*), suggest that these cells have a further relevance in reflecting genetically determined differences in susceptibility to TCDD toxicity.

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