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INDUCTION OF 7-ETHOXYCOUMARIN O-DEETHYLASE ACTIVITY IN CULTURED HUMAN EPITHELIAL CELLS BY 2,3,7,8-TETRACHLORO-DIBENZO-P-DIOXIN (TCDD): EVIDENCE FOR TCDD RECEPTOR

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Summary: The responsiveness of 5 human squamous cell carcinoma (SCC)[1] lines derived from tumors of the epidermis and tongue to 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD) was assessed by measuring the induction of the cytochrome P_1-450-mediated monooxygenase activity, 7-ethoxycoumarin O-deethylase (ECOD). In 4 of the SCC lines the EC_{50}[2] for this response was approximately 10^{-9}M, whereas in one line the EC_{50} was 10^{-10}M. In each of the less sensitive lines a concentration of 10^{-10}M TCDD elicited less than 5% of the maximal enzyme activity. Specific binding of radiolabeled TCDD was detected in the cytosol fraction from all the SCC lines. The relative amount of receptor measured in each line correlated with maximally-induced ECOD activity. The data indicate that human cell lines derived from a target tissue for TCDD toxicity contain the TCDD receptor and show differential sensitivity to TCDD analogous to the murine strain differences in sensitivity regulated by the Ah locus.

One of the most studied of the biochemical responses evoked by TCDD is the induction of cytochrome(s) P_1-450 and the associated monooxygenase activities, aryl hydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin O-deethylase (ECOD) (1). In certain inbred murine strains, the induction of these enzyme activities is regulated by a single genetic locus (designated the Ah locus) and its putative gene product, the TCDD receptor protein (2-4). Based on the findings from several studies (5-7), 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 ex-

[1] Abbreviations used: AHH, aryl hydrocarbon hydroxylase; ECOD, 7-ethoxycoumarin O-deethylase; PBS, phosphate buffered saline, SCC, squamous cell carcinoma; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

[2] EC_{50} = concentration required to elicit 50% of the maximal response.

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pressed 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 (8).

The presence of the Ah locus in human cells, suggested by findings on the induction of AHH activity in cultured mitogen-activated lymphocytes (9), has not been definitively established. Studies on the induction of AHH activity in cultured lymphocytes (10) and monocytes (11) from monozygotic and dizygotic twins have confirmed a heritable component, but have not been able to distinguish between a monogenic or polygenic mode of inheritance. Chromosome mapping in mouse-human cell hybrids suggested that either the structural or regulatory genes for the induction of AHH activity may be located on human chromosome 2 (12); however, participation of mouse genes in the observed response was not ruled out.

In this study we have examined the responsiveness of cultured human SCC lines derived from tumors of the epidermis (a target tissue for TCDD toxicity) and tongue (13,14) to TCDD as measured by the induction of ECOD activity. We observed differential sensitivity to TCDD in these human cell lines analogous to sensitivity differences regulated by the Ah locus in inbred murine strains (4). The presence of the TCDD receptor, suggested by these findings, was confirmed by sucrose-density gradient analysis.

MATERIALS AND METHODS

Cell culture. SCC lines derived from tumors of the tongue and facial epidermis (13,14) were a generous gift from Dr. James Rheinwald (Dana-Farber Cancer Institute, Boston, MA). The cells were grown in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 5% fetal calf serum (Sterile Systems, Inc., Salt Lake City, UT) in the presence of a lethally irradiated layer of murine 3T3 fibroblasts (15). 3T3 cells were kindly provided by Dr. Robert Rice (Harvard School of Public Health, Boston, MA). Cultures were incubated at 37° in a humidified atmosphere of 5% CO_2 and the growth medium was changed every third day.

Stock solutions of TCDD (KOR Isotopes, Inc., Cambridge, MA) were prepared in dimethyl sulfoxide. The final concentration of organic solvent in the medium did not exceed 0.1%. The purity of TCDD was > 99% as judged by gas chromatography and by high pressure liquid chromatography (16). Special precautions used in the handling of TCDD were as described previously (17).

ECOD assay. Treated cultures were rinsed 3 times with 0.02% EDTA in phosphate buffered saline (PBS) (GIBCO, Grand Island, NY) to remove residual 3T3 cells. The attached epidermal cells were scraped into PBS and centrifuged at 10,000 x g for 5 min. The cell pellet was resuspended in 10mM

Tris-HCl (pH 7.5), sonicated and centrifuged at 10,000 x g for 5 min at 4°. Fifty microliter portions of the supernatant were assayed for ECOD activity in a total volume of 0.5 ml as described previously (18).

Receptor assay. Cells from confluent cultures were harvested and washed as described above. The cell pellet was resuspended in 1.5 ml of HEDG buffer (25 mM Hepes; 1.5 mM EDTA; 1.0 mM dithiothreitol; 10% glycerol, pH 7.6), sonicated and centrifuged at 100,000 x g for 1 h. Five hundred microliter portions of the supernatant were incubated with 1 nM [^{3}H]TCDD alone, or with radiolabel plus a 100-fold molar excess of unlabelled TCDD for 30 min at 20° in 12 x 75 mm silanized disposable glass test tubes (19). The tubes were then placed in an ice bath for 5 min. Bound [^{3}H]TCDD was measured by adding the samples to dextran-charcoal pellets (10 mg of charcoal, 1 mg of dextran) (20). The dextran-charcoal pellet was resuspended and the sample was incubated for 15 min at 4° (20). The mixture was then centrifuged at 3000 x g for 5 min and 300 µl of the supernatant (equivalent to 2-5 mg protein) was layered on top of a linear 5% to 20% sucrose gradient prepared in HEDG buffer. The gradients were centrifuged at 235,000 x g for 16 h and 0.2 ml fractions were collected. Five milliliters of ACS scintillation cocktail (Amersham Corp., Arlington Heights, IL) were added and the radioactivity was measured in a Beckman LS-7000 scintillation counter. [^{14}C]-Methylated bovine serum albumin (kindly provided by Dr. Robert Rice, Harvard School of Public Health, Boston, MA) was added to the sample as an internal sedimentation marker.

Protein. Protein concentration was estimated using the dye-binding assay described by Bradford (21) with bovine serum albumin as the standard. Protein dye binding reagent was obtained from Bio-Rad Laboratories (Richmond, CA).

RESULTS

The log dose-response curves for the induction of ECOD activity by TCDD in confluent cultures of SCC cells are shown in Fig. 1. The greatest maximally induced activity was measured in cell lines SCC-13 and SCC-9. TCDD produced an intermediate response in line SCC-15 and the smallest response in lines SCC-4 and SCC-12F.

Absolute enzyme activities were converted to fractional responses (maximally-induced activity = 1.0) to compare the sensitivity of the SCC lines to TCDD (Fig. 2). In 4 of the cell lines, SCC-4, 9, 12F and 13, the EC_{50} values for the induction of ECOD activity were approximately 1 nM (range: 0.8-2.0 nM), whereas in one line, SCC-15, the EC_{50} value was 0.1 nM (Table I). In each of the less sensitive lines a concentration of 0.1 nM TCDD elicited less than 5% of the observed maximal enzyme activity (Fig. 2).

The potency of TCDD as an inducer of ECOD activity in the SCC lines (Fig. 2 and Table 1) suggested that this response was receptor mediated. The presence of the TCDD receptor was confirmed using sucrose-density gradient analysis (Table II). Under the given assay conditions using a

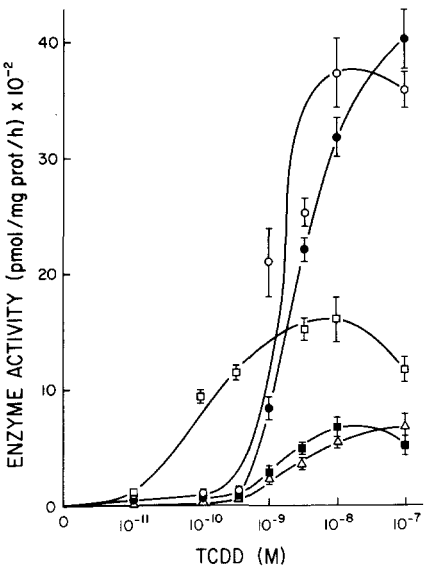

Figure 1. Log dose-response curves for the induction of ECOD activity. Medium containing the indicated concentration of TCDD was added to confluent cultures and ECOD activity was assayed as described in Materials and Methods. Values shown represent the mean ± SE of quadruplicate cultures. SCC-9 (O——O); 13 (●——●); 15 (□——□); 12F (■——■); 4 (Δ——Δ).

concentration of 1 nM [^{3}H]TCDD (estimated to produce 85 to 90% saturation of specific binding sites, as calculated from the reported K_D value in murine liver; ref. 22), the greatest amount of specific binding was measured in line

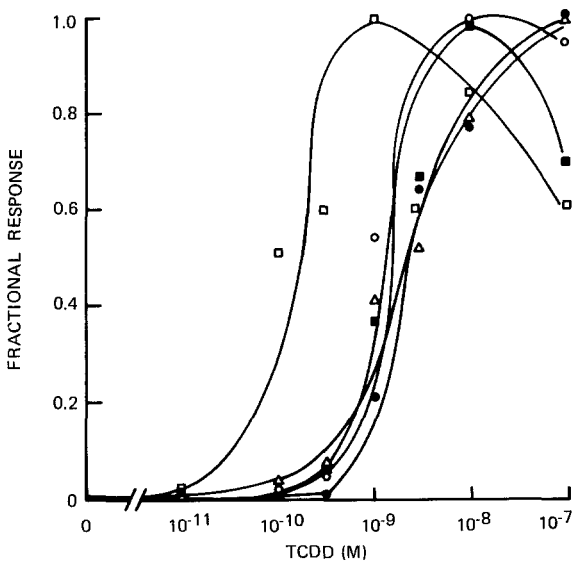

Figure 2. Fractional log dose-response curves. The data shown in Fig. 1 were converted to fractional responses by equating maximally induced activity to 1.0 and control activity to 0. SCC-9 (O——O); 13 (●——●); 15 (□——□); 12F (■——■); 4 (Δ——Δ).

Table I. Sensitivity of SCC lines to induction of ECOD activity by TCDD

Cell Line	EC_{50}[a] (nM)	ECOD Activity[a] (pmol/min/mg protein) Control	Maximally Induced
SCC-15	0.1	0.5 ± 0.1	26 ± 3
SCC-12F	0.8	0.8 ± 0.2	12 ± 2
SCC-13	1.2	ND[b]	67 ± 5
SCC-9	1.3	0.2 ± 0.1	62 ± 5
SCC-4	2.0	0.1 ± 0.1	12 ± 2

[a]Calculated from the dose-response curves shown in Fig. 1. Values represent the mean ± SE for quadruplicate cultures.

[b]ND, not detectable (< 0.01 pmol prod/min/mg protein).

SCC-9, intermediate binding in line SCC-15, and the lowest binding in line SCC-12F (Table II). The relative amount of receptor measured in each line correlated with the maximally-induced response to TCDD (Table II).

DISCUSSION

In this report we have shown that cultured human SCC lines respond to TCDD as measured by the induction of ECOD activity, a cytochrome P_1-450-mediated monooxygenase. In 4 of the 5 cell lines studied, the EC_{50} for this

Table II. Specific binding of [^{3}H]TCDD to cytosol fractions from SCC Lines[a]

Cell Line	Specific Binding (fmol/mg cytosol protein)	Relative Response Specific Binding[b]	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 in Materials and Methods. Values shown represent the average from two separate experiments. The range is given in parenthesis. 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 fmol/mg cytosol protein, respectively.

[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 Table I and normalized with respect to the value obtained for line SCC-12F.

response was approximately 1 nM, whereas in one line (SCC-15), the EC_{50} was 0.1 nM (Table I). The 10-fold difference in the sensitivity of the human SCC lines to TCDD, as indicated by comparing EC_{50} values (Table I), is identical to that reported for inbred murine strains in which differential sensitivity to TCDD is determined by the Ah locus (7). The available biochemical and genetic data indicate that the murine Ah locus codes for a cytosolic receptor protein for TCDD and isosteric analogs (8,23). We identified a specific binding species for TCDD in the cytosolic fractions from each of the SCC lines (Table II). A striking correspondence was found between the relative amounts of receptor measured and the magnitude of maximally-induced ECOD activity (Tables I and II). Thus it appears that the human SCC lines examined in this report possess the equivalent of the murine Ah locus which regulates the expression of TCDD-inducible monooxygenase activity.

Responses to TCDD regulated by the Ah locus in the mouse include: (1) the coordinate expression of a battery of inducible enzymes including cytochrome(s) P_1-450 (4); (2) cleft palate formation and thymic atrophy (5); and (3) epidermal hyperplasia in murine strains containing the hr locus (6). In the murine teratoma cell line, XB-2, TCDD has been shown to produce a hyperkeratinization response which appears to be mediated through a cytosolic receptor (24).

The extent of biochemical and toxic responses regulated by the TCDD receptor in human SCC lines is not known; however, in certain lines TCDD alters cell proliferation and differentiation as judged by colony expansion and keratinization (25). In at least one cell line (SCC-12F) TCDD inhibits colony expansion and down-regulates the specific binding and uptake of epidermal growth factor (L. G. Hudson et al., manuscript in preparation). The role of the TCDD receptor in the regulation of these events is suggested by dose-response parameters and structure-activity relationships (25). With the direct demonstration of the receptor in these cells (Table II), it should be possible to elucidate the mechanisms by which this protein controls cell proliferation and differentiation.

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