

TCDD-mediated alterations in the AhR-dependent pathway in Seveso, Italy, 20 years after the accident

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Approximately 20 years after the Seveso, Italy, accident we conducted a population-based study to evaluate the impact of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure on cancer using mechanistically based biomarkers of dioxin response in humans. TCDD toxic effects are mediated by the aryl hydrocarbon receptor (AhR). We studied the AhR-dependent pathway in lymphocytes from 62 subjects randomly sampled from the highest exposed zones and 59 subjects from the surrounding non-contaminated area, frequency matched for age, gender and smoking. To our knowledge, this is the most comprehensive investigation to date designed to evaluate the key genes in the pathway, including AhR, aryl hydrocarbon receptor nuclear translocator, CYP1A1 and CYP1B1 transcripts and CYP1A1-associated 7-ethoxyresorufin *O*-deethylase (EROD) activity in a population heavily exposed to dioxin. Current lipid-adjusted plasma TCDD concentrations in these subjects ranged from 3.5 to 90 ng/kg (or p.p.t.) and were negatively associated with AhR mRNA in unstimulated peripheral blood mononuclear cells ($P = 0.03$). When mitogen-induced lymphocytes were cultured with 10 nM TCDD, all AhR-dependent genes were induced 1.2- to 13-fold. In these cells, plasma TCDD was associated with decreased EROD activity. In addition, there was a strong positive correlation between AhR and CYP1A1 expression ($P = 0.001$) and between AhR and CYP1B1 expression ($P = 0.006$). CYP1A1 expression was also strongly correlated with EROD activity ($P = 0.001$). The analysis of the expression of dioxin-inducible genes involved in carcinogenesis may help in determining dose-response relationships for human exposure to dioxin *in vivo* and in assessing the variability of human response, which

may indicate the presence of subjects more susceptible to disease as a result of such exposures.

Introduction

In 1997, the International Agency for Research on Cancer established that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a human carcinogen (1) based on a plausible mechanism involving the highly conserved aryl hydrocarbon receptor (AhR), animal models and human data from industrial exposures and accidents. However, the ability to assess risk to the public has been hampered because most of the existing human cohorts are primarily males with mixed exposures in occupational settings.

In 1976, an industrial accident exposed several thousand people to substantial quantities of TCDD in Seveso, Italy. The exposed population consisted of both genders, a wide distribution of ages and a broad range of exposures and therefore provides a unique opportunity to study the effect of dioxin in a non-occupational setting. Three contamination zones (A, B and R) were delimited. A zone-based cohort including nearly 300 000 subjects living in these zones as well as in the surrounding non-contaminated area has been followed for mortality and cancer incidence studies (2,3). An increase in cancer incidence of (3,4) and mortality from (2) lymphohemopoietic neoplasms has been reported.

Approximately 20 years after the exposure, we designed a population-based study to evaluate the impact of TCDD exposure upon mechanistically based biomarkers of dioxin response in humans. We randomly selected the study's subjects from the most exposed zones (A and B) and from the surrounding non-contaminated zone in order to estimate TCDD plasma levels and gene expression in the general population of the entire area. In individuals from zones A and B, elevated plasma TCDD levels [ranging from background values to 90 ng/kg lipid, or parts per trillion (p.p.t.)] were still present after a period of time roughly equivalent to two biological half-lives, with significantly higher levels in women (5,6). In contrast, other dioxin-like congeners were at background levels in both TCDD-exposed and non-exposed areas. TCDD levels in study subjects were within the range of body burdens associated with sensitive dioxin-dependent responses in animal studies, such as induction of CYP1A1 (7).

Genetic and biochemical studies indicate that the AhR is necessary for most of the toxic effects of TCDD, such as tumor promotion, thymic involution, craniofacial anomalies, skin disorders and alterations in the endocrine, immunological and reproductive systems (8,9). TCDD-activated AhR can induce changes in growth factor receptor signaling, cytosolic signaling proteins, calcium mobilization, tumor suppressor proteins and oncogene or cell cycle proteins (10–12) and can form a complex with the retinoblastoma protein (9,13) or the

Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; BMI, body mass index; CI, confidence interval; EROD, 7-ethoxyresorufin *O*-deethylase; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; IS, internal standard; PBS, phosphate-buffered saline; p.p.t., parts per trillion; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TEF, toxic equivalency factor; TEQ, toxic equivalent.

RelA NF- κ B subunit (14). AhR has been known for a long time as a ligand-activated receptor and transcription factor that forms an active heterodimer with the aromatic hydrocarbon nuclear translocator (ARNT/HIF-1 β) and activates the transcription of xenobiotic metabolizing enzymes, such as cytochrome P4501A1 (CYP1A1) and P4501B1 (CYP1B1) as well as other genes (15). Prolonged expression of CYP1A1 may increase the likelihood of deleterious DNA lesions, due to an increase in the generation of genotoxic metabolites and reactive oxygen species (18). Polymorphisms of the CYP1A1 gene and the magnitude of induction of CYP1A1 gene expression by AhR agonists in mitogen-activated human lymphocytes have been correlated with an increased risk of lung cancer in some studies (19), but not in others (20). Similarly, CYP1B1 may be involved in the mechanism of carcinogenesis through its metabolism of 17 β -estradiol and bioactivation of polycyclic aromatic hydrocarbons and arylamines (21,22). Both CYP1A1 (23) and CYP1B1 (24) mRNA levels, measured by quantitative RT-PCR in peripheral lymphocytes, have been proposed as biomarkers of TCDD biological effective dose in humans.

We report here results on the measurement of expression of several genes involved in the AhR pathway, specifically AhR, ARNT, CYP1A1 and CYP1B1 and CYP1A1-associated 7-ethoxyresorufin *O*-deethylase (EROD) activity in study subjects' peripheral blood lymphocytes. Some AhR-dependent markers, such as CYP1A1 expression and EROD activity, are known to be only barely detectable in uncultured lymphocytes (25,26). Therefore, we measured the AhR-related markers in both unstimulated cells and in lymphocytes treated with mitogen and *in vitro* TCDD. The main objective of the study was to verify whether plasma levels of dioxin, measured approximately two decades after the accident, were associated with this pattern of AhR-dependent gene expression and activity and to identify environmental or host factors which could modify such an association.

Materials and methods

Study population

The study subjects were recruited between December 1992 and March 1994. Sixty-two subjects were randomly sampled from the highest exposed zones (A and B) and 59 subjects from the surrounding non-contaminated area (non-ABR), frequency matched for age, gender and smoking, as previously described (6). Informed consent was obtained from participants and the study was reviewed and approved by the local Institutional Review Board. Residence in the specific zone (A, B or non-ABR) was established by determining address and verified by establishing actual domicile and presence in the specified area at the time of the accident. A questionnaire including data on demographics, smoking status and number of cigarettes smoked the day before the study, foods consumed at the time of the accident, residential history, occupation and reproductive and medical history was administered by trained interviewers. Subjects with severe medical illness (liver, kidney, cardiac, immune, neoplastic or major psychiatric disease) were excluded through telephone calls assisted by a physician. Exclusion rates were low and similar across the zones (five from zone non-ABR and four from zone B).

Measurement of TCDD in plasma

The dioxin measurements in human plasma were performed at the CDC using a high resolution gas chromatography/high resolution mass spectrometry analysis (27). Specifically, TCDD and 21 other dioxin or dioxin-like congeners were measured, including 10 dibenzofurans, four co-planar polychlorinated biphenyls and seven additional dibenzo-*p*-dioxins. Results are reported in p.p.t., lipid adjusted. Of the 121 subjects, 11 samples (four from zone B and seven from zone non-ABR) were inadequate and were excluded from the analyses based on plasma TCDD. In another 23 subjects (nine from zone B and 14 from zone non-ABR) levels were determined to be below the detection threshold and so values were estimated by dividing the lipid-adjusted detection

limit by $\sqrt{2}$ (28). Excluding or assigning 0 values for these samples did not substantially change the reported findings. The toxic equivalent (TEQ) for a mixture of 'dioxin-like compounds' is defined as the product of the concentration of each congener multiplied by its specific toxic equivalency factor (TEF). The TEF of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (29–31) was defined as the toxic potency of the individual congener relative to TCDD, which is assigned a TEF of 1.0.

Biological sample acquisition and storage

Donors provided 5–50 ml of whole blood, which was collected into tubes treated with sodium heparin. The blood was diluted with Hank's balanced salt solution (HBSS) (Life Technologies, Gaithersburg, MD) at a proportion of 17 ml blood per 13 ml salt solution. Mononuclear cells were separated by Ficoll Hypaque density gradient centrifugation at 1000 *g* (Histopaque 1077; Sigma Chemical Co., St Louis, MO). The buffy coat containing the mononuclear cells was washed with 25 ml basal lymphocyte culture medium (basal medium) consisting of RPMI 1640 (Life Technologies) with 10% sterile filtered fetal bovine serum (FBS) (Hyclone, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin and 0.25 μ g/ml amphotericin (Life Technologies). After centrifugation at 250 *g* for 10 min, the pellet was washed in 45 ml HBSS. The cell concentration was adjusted to 2×10^7 cells/ml and an equal volume of freeze medium (RPMI 1640; Life Technologies) with 7.5% cell culture grade DMSO (American Type Culture Collection, Rockville, MD), 20% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μ g/ml amphotericin (Life Technologies) was added. A 1 ml aliquot of cells was frozen at a rate of 1°C/min and then stored in the vapor phase of liquid nitrogen.

Peripheral blood mononuclear cell culture

Mononuclear cells were thawed in a 37°C water bath and washed in 40 ml of basal medium at 37°C. After centrifugation at 200 *g* for 10 min, the cells were resuspended in 1 ml fresh stimulation medium consisting of basal medium supplemented with 1.25 μ g/ml phytohemagglutinin (Murex Diagnostics, Norcross, GA), 0.15% (v/v) pokeweed mitogen (Life Technologies) and 50 μ M 2-mercaptoethanol (Sigma). Treated cells received stimulation medium containing 20 nM TCDD-treated FBS. The method for incorporating and verifying the concentration of TCDD is detailed in Spencer *et al.* (24) and Tucker *et al.* (32). Stimulation medium was added until the cells were at a density of 2×10^6 /ml. Half of each cell suspension was transferred to each of two culture flasks. One flask was treated by adding an equal volume of stimulation medium containing 20 nM TCDD for a final concentration of 10 nM TCDD. The control culture flask received an equal volume of stimulation medium without the TCDD.

Cells were cultured in a 37°C incubator at 95% relative humidity and 5% CO₂. After 72 h (\pm 2 h), the cells were removed and resuspended in stimulation medium. Fourteen millilitres of the resuspension solution was used for subsequent RNA analysis. The remaining cells were pelleted by centrifugation at 300 *g* for 10 min at room temperature and resuspended in 3 ml phosphate-buffered saline (PBS) containing 1 mM EGTA. Cell concentrations were adjusted to 20×10^6 cells/ml in PBS/EGTA. Of this, 200 μ l (2×10^6 cells) was removed for the EROD assay.

The cell suspension intended for RNA analysis was centrifuged at 300 *g* for 10 min and the pellet was resuspended in 1 ml Tri-reagent (Sigma) at room temperature. The Tri-reagent containing the mononuclear cell lysate was stored at -70°C until removal for total RNA isolation.

RNA isolation

Total RNA was isolated from mononuclear cells before and after incubation at 37°C. Tri-reagent, which employs an acid-phenol-guanidine thiocyanate procedure, was used to isolate total RNA as per the manufacturer's instruction. Briefly, samples were centrifuged, extracted with chloroform and washed with isopropanol and 75% cold ethanol. After the addition of 100 μ l DEPC-treated water, the RNA concentration was measured at 260 nM, diluted to 40 ng/ μ l and stored at -70°C.

Quantitative competitive reverse transcription PCR (RT-PCR)

RT-PCR was accomplished by titrating 100 ng test RNA against varying but known concentrations of a heterologous recombinant internal standard (IS) as previously described (23,24). The recombinant IS RNA consisted of a spacer sequence derived from the human *GSTM1* gene. The *GSTM1* spacer was flanked by target RNA-specific forward and reverse primer sites and a reverse transcriptase primer site. A unique IS was constructed for each target RNA. Table I shows the primers used to detect the genes and their associated GenBank listings. Details of the recombinant IS construction, amplification and purification are described in Spencer *et al.* (24).

Depending on the gene, each reverse transcription reaction contained the following concentrations and quantities: 100 ng test RNA, a quantity of IS RNA in a range appropriate for the anticipated expression level, 2–7.5 mM

Table I. Quantitative RT-PCR assay primers and conditions

Gene	GenBank ID	Sequence	RT primer (μM)	RNasin (U/ μl)	Primer (μM)	Mg ²⁺ (mM)	Taq (U/ μl)	Annealing temp ($^{\circ}\text{C}$)	Cycles (<i>n</i>)	
AhR	L19872	forward primer (5' → 3')	gga ctt ggg tcc agt cta atg cac	1.25	1	0.5	2.0	0.03	60	35
		reverse primer (5' → 3')	agc cag gag gga act agg att gag							
		RT primer (5' → 3')	aga ttc gtt gaa							
		mRNA target amplicon (bp)	298							
		IS target amplicon (bp)	204							
ARNT	M69238	forward primer (5' → 3')	ccc tag tct cac caa tcg tgg atc	1	0.75	0.5	3.0	0.025	60	35
		reverse primer (5' → 3')	gta gct gtt gct ctg atc tcc cag							
		RT primer (5' → 3')	cca ata gtt cta							
		mRNA target amplicon (bp)	240							
		IS target amplicon (bp)	151							
CYP1A1	K03191	forward primer (5' → 3')	ggc aga tca acc atg acc aga	1.25	1	0.5	2.0	0.03	65	35
		reverse primer (5' → 3')	agt cta ggc ctc agg gct ctc							
		RT primer (5' → 3')	gcc aga tca gtc							
		mRNA target amplicon (bp)	334							
		IS target amplicon (bp)	265							
CYP1B1	U03688	forward primer (5' → 3')	ggt ctt gag gca ctg cga aag cct	1	0.75	0.5	7.5	0.025	65	40
		reverse primer (5' → 3')	tca taa agg aag gcc agg aca tag							
		RT primer (5' → 3')	ggt gtt ggc agt							
		mRNA target amplicon (bp)	338							
		IS target amplicon (bp)	222							
Actin	M10278	forward primer (5'-3')	aaa cta cct tca act cca tc	1.25	1	0.6	2.0	0.03	54	25
		reverse primer (5'-3')	atg atc ttg atc ttc att gt							
		RT primer (5'-3')	tgg aag gtc gac							
		mRNA target amplicon (bp)	165							
		IS target amplicon (bp)	252							

MgCl₂ (Promega, Madison, WI), NEB buffer [16.6 mM NH₄SO₄, 5 mM 2-mercaptoethanol, 6.8 μM EDTA, 67 mM Tris-HCl, pH 8.8, 0.1 mg/ml bovine serum albumin (BSA) (Sigma)], 1 mM deoxyribonucleoside triphosphate (Promega), 15–20 U recombinant RNasin RNase inhibitor (RNase), 80 U Moloney murine Leukemia virus reverse transcriptase and 1–1.25 μM RT primer (Bioserve Biotechnologies, Laurel, MD) in a final volume of 20 μl . Reverse transcription was performed in a Perkin Elmer 9700 thermocycler. Samples were heated to 37°C for 15 min followed by 5 min at 99°C.

After reverse transcription, 30 μl of PCR product was added to each tube with final concentrations of 0.5–0.6 μM for each of the forward and reverse primers (Bioserve), NEB buffer and 1.25 U *Taq* DNA polymerase (Promega) per reaction tube. The reactions were heated to 94°C for 4 min and then cycled at 94°C for 30 s, from 54 to 65°C for 30 s (the temperature varied according to the specific gene) and 75°C for 30 s. The number of cycles varied by gene transcript. The optimized conditions for each of the RT-PCR assays are summarized in Table I.

Initially, a series of six 10-fold IS dilutions was performed for each sample in order to determine the approximate range of expression. The quantitation was then repeated using a series of six to seven 2-fold IS dilutions for each

RNA. After amplification by PCR, the test and IS cDNAs were separated by electrophoresis (100 V for 3 h) on a 2% NuSieve 3:1 agarose (FMC-BioProducts, Rockland, ME) gel in 40 mM Tris-acetate, 1 mM EDTA buffer. Gels also contained 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide for subsequent detection. A digitized image of the cDNA was obtained using a Molecular Dynamics fluorimager (Amersham, Piscataway, NJ). The digitized images were analyzed by measuring the intensity of the DNA bands with NIH Image software (NIH Image v.1.61). Quantitation was based upon the series of six or seven IS standard concentrations run for each sample by plotting log₁₀ ratio of band intensity of the test RNA and IS RNA against log₁₀ copies of IS RNA. Linear regression was used to interpolate the number of RNA copies present in each 100 ng of test RNA by calculating the *x*-intercept, which corresponds to the equivalent band intensity and, hence, copy number of the test and IS RNAs.

Ethoxyresorufin deethylase (EROD) assay

An aliquot of 500 μl of EROD buffer [5.0 mM MgSO₄ in 0.1 M KPO₄ with 2.0 mg/ml bovine serum albumin (Sigma)], 20 μg NADPH and 50 pmol ethoxyresorufin in 10 μl DMSO were added to 10⁶ mononuclear cells in

100 µl PBS/EGTA buffer and incubated for 30 min at 37°C. The reactions were stopped by the addition of a volume of methanol equivalent to the total reaction volume (620 µl). The reactions were centrifuged at room temperature for 3 min and transferred to a 96-well plate in triplicate. Each plate also included a negative control (prepared from heat-inactivated mononuclear cells), a positive control (prepared from phenobarbital-induced murine microsomes) and a standard curve of 0.19–100 pmol resorufin/well. Fluorescence was measured on a Perkin Elmer LS-50B fluorescence plate reader with excitation at 550 nm and emission at 585 nm. Activity was expressed as activity per 10⁶ lymphocytes or per mg protein. Results are reported as pmol/min/mg protein throughout the paper. Protein was measured using a bicinchoninic acid assay kit, which includes a BSA standard (Pierce BCA protein assay kit).

Statistical analysis

Differences in gene expression and EROD activity between experiments performed in different cell culture conditions were evaluated using the paired *t*-test. The unpaired Student's *t*-test was used for group comparisons. A Bonferroni correction was used for multiple comparisons. Two-sided *P* values are reported. Logarithmic transformations of all variables were used to improve the fit to a normal distribution. Geometric means and 95% confidence intervals (CI) are reported throughout the manuscript. Body mass index (BMI) was defined as weight in kg/height in m² (kg/m²). Percentage body fat was defined as in Knapik *et al.* (33). Pearson's correlation coefficients (*r*) and Wald test *t* values for simple and multiple regression analyses, respectively, are reported throughout the manuscript. We performed simple and multiple linear regression analyses to assess associations between variables. Independent variables for the regression models in the analyses of uncultured cells included: plasma TCDD, related AhR pathway markers, age, gender, actin expression and date of culture assay. For the cultured cells, the independent variables included: actin expression, post-culture viability, batch of experiment (17 batches were categorized in four consecutive groups for the analyses), percentage cell growth, plasma TCDD and related AhR pathway markers. All uncultured cells with viability <75% were excluded from the analyses. Results in 'cultured cells' indicate results from lymphocytes cultured with mitogen and 10 nM TCDD, unless otherwise specified. All analyses were performed with the use of the Stata statistical package Release 7.0 (Stata Corp., College Station, TX).

Results

AhR-dependent markers in different cell conditions

Uncultured cells. In uncultured cells, only AhR, CYP1B1, ARNT and actin expression could be detected. Mean mRNA levels per µg total RNA were 11.9 × 100 000 copies for AhR, 4.7 × 100 000 copies for ARNT and 1.1 × 100 000 copies for CYP1B1 (Table II). Women had lower levels of gene expression, particularly of the AhR gene (Table II), even after adjustment for age, actin and date of assay (*t* = 2.68, *P* = 0.009). However, when plasma TCDD was added to the model, the association between gender and AhR was no longer significant (*t* = 1.52, *P* = 0.13). Biomarker mRNA levels increased with age (Table II), with a statistically significant association in the univariate analysis (*r* = 0.33, *P* = 0.002, *r* = 0.26, *P* = 0.03 and *r* = 0.33, *P* = 0.003 for AhR, ARNT and CYP1B1, respectively) and for AhR and CYP1B1 in the multivariate models (*t* = 2.27, *P* = 0.027 and *t* = 2.51, *P* = 0.015 for AhR and CYP1B1, respectively, models adjusted for plasma TCDD levels, gender, actin, smoking and date of assay). AhR expression was higher in zone non-ABR than in zones A + B (Table II). This association was significant in the multivariate analysis (*t* = 0.16, *P* = 0.03).

Cultured cells. All markers, including AhR, CYP1A1 and CYP1B1 expression and EROD activity, were highly induced when cells were cultured with mitogen and with mitogen + 10 nM TCDD (Table II). We did not measure ARNT expression in cultured cells because too few cells were available for the assay. In addition, ARNT has been shown to be poorly inducible in culture (34,35). For AhR and CYP1B1, the ratio

of the expression in mitogen-stimulated cells compared with expression in uncultured cells (3.0- and 16.6-fold for AhR and CYP1B1, respectively) was larger than the ratio of the expression in cells cultured with mitogen + TCDD compared with mitogen-cultured cells (1.2- and 3.8-fold for AhR and CYP1B1, respectively) (*P* < 0.001). As expected, CYP1A1 mRNA levels and EROD activity were strongly induced (8.8- and 13.1-fold, respectively, *P* < 0.001) when 10 nM TCDD was added to the mitogen-stimulated cells, particularly in subjects with low TCDD exposure or resident in zone non-ABR, in the youngest and in current smokers (Table II).

Plasma TCDD and TEQ by zone

In subjects with detectable results in uncultured lymphocytes, mean plasma TCDD levels were 54.6 p.p.t. in subjects from zone A (*n* = 6, 95%CI = 22.5–132.9), 11.3 p.p.t. in subjects from zone B (*n* = 35, 95% CI = 8.0–16.1) and 4.2 p.p.t. in subjects from the surrounding non-contaminated area (*n* = 35, 95% CI = 3.4–5.4). There was a strong correlation between TCDD and TEQ levels overall (*n* = 76, *r* = 0.86, *P* < 0.0001) and within zones (*n* = 41, *r* = 0.88, *P* < 0.0001 and *n* = 35, *r* = 0.73, *P* < 0.0001 in zones A + B and non-ABR, respectively). The percentage of TEQ due to TCDD was 26% overall (range 10–84%) and varied by zone: it was 35% in zone A + B (range 12–84%) and 18% in zone non-ABR (range 10–41%).

Association between dioxin exposure and AhR-dependent biomarkers

Uncultured cells. In the univariate analysis, AhR (in all cell conditions) and ARNT (in uncultured cells) were lower in subjects with higher exposure (higher TCDD or TEQ plasma levels or resident in zones A + B) (Table II). In mitogen-stimulated cells, CYP1A1 expression was higher in subjects with higher TCDD levels.

In the multivariate model, the negative association between TCDD plasma levels and AhR was statistically significant in uncultured lymphocytes (*t* = -2.28, *P* = 0.026, model adjusted for age, gender, actin and date of assay) (Table III). The association between TEQ levels and AhR was also negative, but not significant in the multivariate model (*t* = -0.73, *P* = 0.467). TCDD was not significantly associated with ARNT (*P* = 0.21) and CYP1B1 (*P* = 0.60) expression.

When we divided the subjects by zone of residence, the association between TCDD and AhR expression (*t* = -0.89, *P* = 0.38 and *t* = -1.50, *P* = 0.15 in zones A + B and non-ABR, respectively) and between TEQ and AhR expression (*t* = -0.34, *P* = 0.74 and *t* = -0.81, *P* = 0.43 in zones A + B and non-ABR, respectively) were still negative, but did not reach statistical significance.

Cultured cells. In cells cultured with mitogen and *in vitro* TCDD, the association between TCDD or TEQ plasma levels and AhR, CYP1A1 or CYP1B1 expression was not significant (Table III), after adjustment for actin expression, post-culture viability, experiment group and cell growth. Although based on a small number of subjects, there was a positive association between plasma TCDD and CYP1A1 expression in zones A + B (*n* = 33, *t* = 2.06, *P* = 0.05). Since CYP1A1 expression was correlated with BMI (*r* = -0.24, *P* = 0.03) and with percentage body fat (*r* = -0.23, *P* = 0.03), we added these variables to the regression model, with no substantial change in the results. In subjects from zones A + B there was also a significant positive association between TEQ and AhR expression (*t* = 2.53, *P* = 0.02).

Table II. AhR-dependent gene expression and EROD activity (geometric means and 95% confidence intervals) by subject characteristics and dioxin exposure

	Uncultured cells ^a				Mitogen-stimulated cells				Mitogen + 10 nM TCDD-stimulated cells			
	AhR ^b	ARNT ^b	CYP1B1 ^b	AhR ^b	CYP1A1 ^b	CYP1B1 ^b	EROD ^c	AhR ^b	CYP1A1 ^b	CYP1B1 ^b	EROD ^c	
All subjects	11.9 (9.9–14.3)	4.7 (3.8–5.7)	1.1 (0.7–1.6)	35.2 (31.4–39.4)	6.0 (5.1–6.9)	18.3 (15.6–21.5)	0.12 (0.09–0.16)	42.8 (39.1–46.9)	52.5 (45.5–60.5)	69.1 (58.0–82.3)	1.57 (1.33–1.86)	
Plasma TCDD												
1.0–7.9 p.p.t.	14.5 ^d (11.2–18.6)	5.5 (4.3–7.0)	1.0 (0.5–2.0)	39.0 ^d (33.0–46.0)	5.4 (4.5–6.5)	18.8 (14.8–23.7)	0.14 (0.10–0.19)	44.3 (38.5–51.0)	50.3 (40.9–61.9)	73.1 (57.2–93.5)	1.89 ^d (1.51–2.38)	
8.0–89.9 p.p.t.	9.1 ^d (6.7–12.2)	3.6 (2.6–5.1)	1.0 (0.6–1.8)	30.9 ^d (26.3–36.4)	7.1 (5.5–9.2)	16.3 (12.7–21.1)	0.11 (0.07–0.19)	41.7 (36.4–47.9)	54.0 (43.6–67.0)	57.9 (44.8–74.8)	1.22 ^d (0.91–1.63)	
Total plasma TEQ												
7.4–37.9 p.p.t.	12.4 (9.8–15.8)	5.3 (4.2–6.8)	0.7 (0.4–1.3)	38.0 (31.8–45.2)	5.8 (4.9–6.8)	18.8 (14.4–24.5)	0.14 (0.10–0.20)	43.2 (37.6–49.6)	50.5 (41.5–61.5)	68.0 (52.0–88.9)	1.92 ^d (1.56–2.34)	
38.0–181 p.p.t.	11.2 (8.2–15.4)	4.0 (2.8–5.6)	1.6 (0.9–2.8)	32.4 (27.9–37.8)	6.7 (5.1–8.8)	16.8 (13.7–20.7)	0.11 (0.07–0.18)	43.0 (37.6–49.3)	54.4 (43.4–68.2)	64.5 (51.8–80.3)	1.27 ^d (0.95–1.71)	
Zone of residence												
Non-ABR	13.7 (10.1–18.4)	4.9 (3.6–6.6)	1.2 (0.6–2.2)	39.3 (32.8–47.1)	5.0 ^d (4.0–6.3)	18.8 (15.0–23.7)	0.12 (0.09–0.16)	45.4 (39.0–52.9)	57.9 (47.9–70.0)	79.6 (60.8–104.0)	1.81 (1.48–2.21)	
A+B	10.5 (8.4–13.2)	4.5 (3.4–5.9)	1.0 (0.6–1.7)	32.3 (27.9–37.3)	6.9 ^d (5.7–8.3)	17.9 (14.2–22.6)	0.12 (0.08–0.19)	41.0 (36.6–46.0)	48.4 (39.1–59.8)	61.6 (48.8–77.9)	1.37 (1.04–1.80)	
Gender												
Male	14.5 ^d (11.8–17.9)	5.6 (4.4–7.0)	1.2 (0.7–2.1)	35.5 (30.5–41.3)	6.4 (5.3–7.7)	18.7 (14.9–23.4)	0.17 ^d (0.12–0.23)	43.6 (38.7–49.1)	54.5 (44.1–67.4)	68.5 (54.0–86.9)	1.75 (1.32–2.32)	
Female	9.6 ^d (7.1–12.9)	3.9 (2.8–5.4)	1.0 (0.5–1.7)	34.9 (29.2–41.6)	5.5 (4.3–6.9)	17.9 (14.1–22.7)	0.09 ^d (0.06–0.13)	42.0 (36.3–48.5)	50.2 (41.3–61.0)	69.8 (53.2–91.5)	1.41 (1.16–1.70)	
Age												
20–45 years	9.4 ^d (7.1–12.5)	3.9 ^e (2.7–5.7)	0.7 ^f (0.4–1.3)	34.3 (28.4–41.4)	4.9 ^d (4.1–6.0)	18.4 (14.5–23.2)	0.08 ^d (0.06–0.12)	39.4 (34.0–45.7)	56.2 (45.0–70.3)	71.9 (54.1–95.7)	1.58 (1.25–2.00)	
46–76 years	14.5 ^d (11.4–18.3)	5.3 ^e (4.4–6.5)	1.4 ^f (0.8–2.5)	36.1 (31.4–41.5)	7.1 ^d (5.7–8.8)	18.3 (14.6–23.0)	0.17 ^d (0.12–0.25)	46.5 (41.8–51.7)	49.1 (40.8–59.2)	66.5 (53.3–83.0)	1.55 (1.21–2.01)	
Current smoker												
No	12.7 (10.3–15.7)	4.8 (3.8–6.0)	1.2 (0.8–1.9)	34.0 (30.1–38.4)	6.2 (5.2–7.3)	18.3 (15.3–21.9)	0.14 (0.10–0.18)	42.2 (38.0–46.8)	50.1 (42.1–59.6)	70.3 (57.9–85.3)	1.47 (1.21–1.79)	
Yes ^g	9.5 (6.5–14.1)	4.2 (2.6–6.8)	0.7 (0.3–1.7)	39.4 (29.5–52.5)	5.3 (3.8–7.4)	18.3 (12.2–27.5)	0.08 (0.04–0.17)	44.9 (36.8–54.8)	61.0 (48.4–77.0)	65.1 (42.0–101.0)	2.00 (1.41–2.85)	

^aSubjects with low pre-culture viability (<75%) were excluded from the analysis.^bCopies × 100 000/μg.^cpmol/min/mg.^d*p* < 0.05, Student's *t*-test for differences between the two categories of the covariate.^e*r* = 0.26, *P* = 0.03, Pearson's correlation between age (continuous variable) and ARNT expression in uncultured cells.^f*r* = 0.33, *P* = 0.003, Pearson's correlation between age (continuous variable) and CYP1B1 expression in uncultured cells.^gSubjects who had smoked at least one cigarette during the 24 h before the study.

Table III. Association between plasma TCDD or TEQ and AhR-dependent markers in uncultured cells and in cells cultured with mitogen and 10 nM TCDD

Correlated variables		Uncultured cells ^a			Mitogen + 10 nM TCDD-stimulated cells ^b		
		<i>n</i>	<i>t</i>	<i>P</i>	<i>n</i>	<i>t</i>	<i>P</i>
Plasma TCDD	AhR	72	-2.28	0.026	56	1.16	0.253
Plasma TEQ	AhR	72	-0.73	0.467	56	1.80	0.078
Plasma TCDD	ARNT	61	-1.27	0.211			
Plasma TEQ	ARNT	61	-0.54	0.589			
Plasma TCDD	CYP1A1				60	-0.05	0.960
Plasma TEQ	CYP1A1				60	0.22	0.827
Plasma TCDD	CYP1B1	68	-0.53	0.596	60	-1.26	0.212
Plasma TEQ	CYP1B1	68	-0.22	0.827	60	-2.00	0.051
Plasma TCDD	EROD				52	-2.61	0.012
Plasma TEQ	EROD				52	-2.20	0.033

All variables were log transformed.

^aSamples with pre-culture viability <75% were excluded. Results adjusted for age, gender, actin expression and date of assay in multivariate regression analysis.

^bResults adjusted for actin expression, post-culture viability, experimental group and cell growth in multivariate regression analysis.

Table IV. Association among markers within the AhR pathway in uncultured cells and in cells cultured with mitogen and 10 nM TCDD

Correlated variables		Uncultured cells ^a			Mitogen + 10 nM TCDD-stimulated cells ^b		
		<i>n</i>	<i>t</i>	<i>P</i>	<i>n</i>	<i>t</i>	<i>P</i>
Ah-R	ARNT	69	4.20	<0.001			
Ah-R	CYP1A1				61	3.62	0.001
Ah-R	CYP1B1	75	4.50	<0.001	61	2.87	0.006
Ah-R	EROD				53	1.90	0.064
ARNT	CYP1B1	67	2.26	0.028			
CYP1A1	CYP1B1				67	1.12	0.266
CYP1A1	EROD				59	3.48	0.001
CYP1B1	EROD				59	1.69	0.098

All variables were log-transformed

^aSamples with pre-culture viability <75% were excluded. Results adjusted for age, gender, actin expression and date of assay in multivariate regression analysis.

^bResults adjusted for actin expression, post-culture viability, experiment group and cell growth in multivariate regression analysis.

Overall, plasma TCDD and TEQ levels (Table III) were negatively and significantly associated with EROD activity ($t = -0.33$, $P = 0.01$ and $t = -2.20$, $P = 0.03$ for TCDD and TEQ levels, respectively).

Association among markers within the AhR-dependent pathway

Uncultured cells. There was a strong correlation between AhR and ARNT gene expression ($t = 4.20$, $P < 0.001$), AhR and CYP1B1 expression ($t = 4.50$, $P < 0.001$) and ARNT and CYP1B1 expression ($t = 2.26$, $P = 0.028$) in the multivariate model adjusted for age, gender, actin and date of assay (Table IV). When AhR and ARNT were fitted in one singular regression model, AhR ($t = 3.30$, $P = 0.002$), but not ARNT ($t = 0.43$, $P = 0.67$), significantly predicted CYP1B1 levels. In addition, there was no significant interaction

between AhR and ARNT in this model. The median ratio between AhR and ARNT was 2.3, with an interquartile range of 1.5–4.0.

Cultured cells. Markers measured in mitogen-treated cells and markers measured in cells cultured with mitogen + TCDD were strongly correlated ($P < 0.0001$). AhR and CYP1B1 expression levels measured in uncultured cells were not significantly associated with the corresponding AhR and CYP1B1 expression levels in TCDD-stimulated cultured cells ($r = 0.01$, $P = 0.93$ for AhR and $r = 0.14$, $P = 0.22$ for CYP1B1). Within cells cultured with mitogen + TCDD, AhR expression was highly and significantly associated with that of the *CYP1A1* ($P = 0.001$) and *CYP1B1* ($P = 0.006$) genes in the multivariate model adjusted for actin expression, post-culture viability, batch of experiment and cell growth (Table IV). In addition, a borderline significant association between AhR expression and EROD activity ($P = 0.06$) was observed. As expected, CYP1A1 expression was highly correlated with the corresponding EROD activity ($P = 0.001$).

Discussion

TCDD is considered a potent carcinogen based on *in vitro* experiments and animal studies (1) and exerts most of its toxic effects through the AhR. To our knowledge, this is the first population-based study that has investigated multiple components of the AhR pathway in lymphocytes from individuals accidentally exposed or not exposed to higher than background levels of TCDD.

TCDD plasma levels were significantly associated with lower levels of AhR expression in uncultured cells. Females, who had higher levels of dioxin in our study (5,6), showed lower AhR transcripts. Although not significantly, TEQ levels were also negatively associated with AhR expression. The negative association between TCDD or TEQ levels and AhR was still evident when we considered subjects within their respective zones of residence, even though the association was not significant, possibly because of the low number of subjects in each category and/or the possible misclassification of the true biological exposure when zone is used as a surrogate for measured TCDD values.

Our findings may suggest that, at least in lymphocytes, long-term presence of dioxin in the human body does not result in an increase in AhR pathway responsiveness or that responsiveness is eventually lost or reduced decades after the initial acute exposure. Both exposed and unexposed study subjects donated their blood at approximately the same time in the morning, thus it is unlikely that a circadian variation of AhR (36) could have affected the results. However, transcript levels may have been influenced by other factors that intervened between the exposure to TCDD and our measurement. Most importantly, in the two decades since the initial exposure, responsiveness may have been lost due to adaptation, i.e. death or alteration of the responsive cell populations. Future studies may evaluate the receptor protein levels in order to determine whether the AhR protein is also reduced.

We did not find a similar negative association between TCDD plasma levels and ARNT expression. This is consistent with animal models, which indicate a stronger TCDD-dependent down-regulation of AhR protein in comparison with that of ARNT, a difference that may be due to the need to conserve ARNT for other signaling pathways (35,37,38).

As previously reported (23,24,39,40), addition of mitogen to the cell culture and *in vitro* TCDD resulted in a significant increase in AhR-dependent gene expression. Measured markers within the AhR pathway were highly correlated with each other. Overall, all gene expression, including that of the AhR gene, was not correlated with plasma TCDD levels. The variation in mRNA levels following stimulation with mitogen and, to a lesser extent, with TCDD in culture may have masked the effect of the accident-related TCDD present in blood. Also, after two half-lives, TCDD levels may have been too low to elicit a strong induction of the AhR pathway.

Plasma TCDD and TEQ levels were associated with decreased EROD activity in cultured cells. Cigarette constituents, such as benzo[*a*]pyrene and nicotine, are known to induce CYP1A1 expression (41) and EROD activity has been shown to be correlated with daily cigarette consumption in surgical lung samples (42). However, recent use of tobacco products was not significantly associated with CYP1A1 mRNA expression in cultured cells, so this variable did not account for the findings. In a previous study (43) we found that EROD activity was significantly higher in subjects carrying a polymorphic variant of the CYP1A1 gene, while expression of the CYP1A1 mRNA did not vary across genotypes. Further studies including genotype data on CYP1A1 and other related genes would be required to clarify this issue.

The observation that overall gene expression in uncultured cells were lower in subjects from zones A + B in comparison with the subjects from the non-contaminated area suggests that the zone classification may reflect the effect of acute exposure to TCDD and the presence of other unknown factors at the moment of the accident. In addition, the percentage of TEQ due to TCDD in zones A + B was almost 2-fold that in zone non-ABR. TCDD may elicit a different effect on gene expression in comparison to the effect due to other congeners. The acute exposure to TCDD may have resulted in a down-regulation of the AhR pathway, death or unresponsiveness of key cells or in a differential co-induction of a repressor molecule (44) or down-regulation of the Ah receptor-interacting protein (45) in the exposed individuals. Moreover, alternative pathways, such as the retinoic acid receptors signal transduction pathway, may have interfered with AhR signaling (46) or with the protein kinase C-mediated events required for the AhR signaling pathway.

We measured all biomarkers in peripheral blood lymphocytes. While TCDD has effects on diverse cell types, lymphocytes are readily available from blood. Mitogen-stimulated lymphocytes express the AhR, CYP1A1 and CYP1B1 genes (23,24,47,48) and similarities in the regulation of lymphocyte CYP1A1 with the liver isoenzyme have been found (49), suggesting that CYP1A1 expression in peripheral blood lymphocytes can be used to monitor hepatic enzyme activity (49). In addition, an excess of lymphoproliferative cancers is a postulated dioxin consequence (2,3), particularly in this population, and peripheral lymphocytes may provide the best feasible surrogate for lymphatic cell populations for epidemiological investigations.

Consistent with effects observed in endometrial cells (50), AhR, together with ARNT and CYP1B1, increased with age in uncultured cells and CYP1A1 increased with BMI and percentage body fat in our study. All the other factors identified in previous studies (5,6) as important determinants of TCDD levels in the population, such as distance from the accident site, consumption of domestic livestock and poultry and

smoking, were not associated with AhR-dependent markers. In contrast, many laboratory-related factors, such as pre- and post-culture cell viability, storage and shipment conditions, cell growth, day of experiment and actin expression, were strongly associated with gene expression and EROD activity. We have adjusted the regression models for the possible confounders, controlled the analyses for multiple comparisons and excluded results obtained in uncultured cells with low viability. The present study highlights the need to experimentally and statistically assess these types of factors for their impact on gene expression results in molecular epidemiology studies.

In conclusion, TCDD plasma levels were associated with a reduction in AhR expression in unstimulated cells. If substantiated, this finding may suggest that long-term exposure to TCDD perturbs AhR pathway regulation. Precisely how this modulation of AhR alters the potency of dioxin as a carcinogen is not known, and will need to be explored. In mitogen-stimulated cells cultured *in vitro* with TCDD, plasma dioxin levels showed a lack of association with AhR-dependent gene expression and a negative association with EROD activity. Gene message levels within the AhR signaling pathway were highly correlated. Larger studies investigating gene and protein interactions in subjects with high TCDD exposure levels are needed to further elucidate TCDD action and possible carcinogenic effects in humans as well as the influence of individual susceptibility on TCDD-related adverse health outcomes.

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