Mechanistically Based Markers of Exposure and Response to Dioxin in Occupationally Exposed Individuals

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Abstract

This study examines biochemical markers in human peripheral blood lymphocytes which may be predictive of dioxin exposure and resulting adverse health outcomes. Methods have been developed for the analysis of gene products involved in the biological response to dioxins in both resting and cultured lymphocytes. Aryl hydrocarbon receptor (AhR), AhR nuclear translocator (ARNT), cytochrome P4501A1 (CYP1A1) and cytochrome P4501B1 (CYP1B1) mRNA levels are measured by quantitative RT-PCR, CYP1A1 protein by enzyme assay, and AhR and ARNT protein by western blot. We are currently examining these markers in populations with varying magnitude and types of exposure to dioxins. One cohort under study consists of 110 workers with potential exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other dioxin-like compounds during the production of phenoxy-herbicides in a German chemical plant. The range of serum TCDD and total dioxin toxic equivalents (TEQ) in these individuals at the time of sampling was 0-600 and 1-915 ppt lipid, respectively. Some, but not all of the individuals with elevated serum dioxin levels developed the skin disorder chloracne. There was a 15-fold variation in TCDD-inducible CYP1A1 activity in a subset of these workers (n=34). Neither serum dioxin concentration (after exposure) nor CYP1A1 activity in this subgroup differentiated between individuals previously diagnosed as having chloracne. Quantification of these markers relative to dioxin body burden and observed health effects will determine which may reflect dioxin exposure and/or predict susceptibility to adverse health effects.

Introduction

TCDD and related compounds, collectively referred to as dioxins, are ubiquitous and persistent environmental contaminant exhibiting toxicity to multiple organ/hormonal systems in humans, laboratory animals, and wildlife [1)]. TCDD is also carcinogenic in several species of laboratory animals and has recently been classified as a human carcinogen [2)]. While all individuals carry a measurable body burden of TCDD and dioxin-like compounds, the response elicited by exposure to TCDD is likely to differ between individuals with similar body burdens of TCDD.

The biochemical response to TCDD involves changes in gene expression mediated by the AhR and the ARNT proteins, which together function as a ligand activated transcription factor [4)]. TCDD influences the expression of a broad array of genes including CYP1A1, CYP1A2, and

CYP1B1 [4]. The spectrum of adverse health effects resulting from dioxin exposure can be at least partially attributed to alterations in expression of TCDD-inducible genes [3]. Since the cellular response to dioxin is dependent on initial binding to the AhR, the individual biological response is mechanistically linked to the AhR. Thus, components of the TCDD signal transduction pathway (i.e. AhR and ARNT) and TCDD-inducible genes (i.e. CYP1A1 and CYP1B1) are potential mechanistically based biochemical markers of exposure and response to dioxins. Human studies have demonstrated an association between exposure to AhR ligands and elevated levels of CYP1A1 [5-8]. Furthermore, previous studies in our laboratory and others have shown variation in AhR levels and dioxin-inducible CYP1A1 enzyme activity within the human population [7,9-11]. Interindividual variation in response to dioxin is likely the cumulative effect of many contributing factors including prior dioxin exposure, genetic makeup, age, diet and smoking, although the mechanistic basis for interindividual variation in response to dioxin is unknown. An integrated approach evaluating all these variables will provide insight into the expected health risks associated with current exposure levels to dioxins.

We have developed methods for the quantitative analysis of AhR, ARNT, CYP1A1, and CYP1B1 expression in human peripheral blood lymphocytes. The objectives of this study were specifically to 1) establish human peripheral blood lymphocytes as a model system for evaluating potential biomarkers relevant to dioxin exposure and response and 2) evaluate AhR, ARNT, CYP1A1, and CYP1B1 expression in peripheral blood lymphocytes from a cohort of German chemical plant workers occupationally exposed to dioxins.

Experimental Methods

Human peripheral blood lymphocyte cell culture. Lymphocytes were isolated by Ficoll separation from freshly drawn heparinized venous blood and cryopreserved. Subsequently, lymphocytes were cultured for 72 hr at $1x10^6$ cells/ml in RPMI 1640 medium with the mitogens phytohemagglutinin (1.25 ug/ml) and pokeweed mitogen (0.15% vol/vol), and with or without 10 nM TCDD.

Assay of CYP1A1 enzyme activity. CYP1A1-dependent EROD activity was performed essentially as described [12]. Freshly harvested cells ($2x10^6$) were suspended in PBS and diluted into EROD assay buffer (0.1 M potassium phosphate, 5 mM magnesium sulfate, 2 mg/ml bovine serum albumin), with 0.81 μM ethoxyresorufin and 38.7 μM NADPH (final concentration). Conversion of ethoxyresorufin to resorufin was measured on a Perkin Elmer fluorescent plate reader with excitation at 550 nm and emission at 585 nm.

Quantitative analysis of mRNA levels by "competitive" RT-PCR. Construction of recombinant RNA competitors competitive RT-PCR were performed as previously described [7,13] with minor modifications. A recombinant internal standard (recIS) RNA consisting of a spacer sequence amplified from the human *GSTM1* gene and flanked by target RNA-specific forward and reverse primer sites and a reverse transcriptase primer site was constructed for each target RNA. Total RNA (100 ng) was reverse transcribed in the presence of increasing amounts of the corresponding recIS RNA. The resulting cDNAs were co-amplified by PCR and analyzed by agarose gel electrophoresis and ethidium bromide staining. The band intensity of PCR products was analyzed on digitized images of electrophoresis gels using NIH Image software. The log ratio of band intensity of the target RNA and recIS RNA was plotted against the log copies of recIS RNA. Linear regression was used to interpolate the number of RNA copies present in each sample which is equal to the x-axis intercept, i.e. where the target and recIS RNA band intensity is equal.

Serum dioxin analysis. Frozen serum separated from peripheral blood collected in 1992 was analyzed for 8 polychlorinated dibenzo-*p*-dioxin (PCDD), 10 polychlorinated dibenzofuran (PCDF), and 4 polychlorinated biphenyl (PCB) congeners by high resolution gas chromatography/mass spectrometry as described [14].

Results and Discussion

Analysis of dioxin biomarkers in human peripheral blood. The general strategy for the evaluation of potential dioxin biomarkers in humans is shown in Fig. 1. Peripheral blood from individuals potentially exposed to dioxin-like compounds was collected and processed for three different purposes. Serum was separated to determine levels of PCDDs, PCDFs, and PCBs. Samples were also collected for DNA isolation and analysis of polymorphisms in the *CYP1A1* and *GSTM1* genes. Lymphocytes were isolated to evaluate mRNA levels of AhR, ARNT, CYP1A1, and CYP1B1 and protein levels of AhR, ARNT, and CYP1A1 in both resting and cultured cells.

A summary of the expression of each of the dioxin biomarkers in peripheral blood lymphocytes from 6-9 local volunteers from North Carolina is given in Table 1. AhR, ARNT, and CYP1B1 mRNA can be detected in resting, uncultured lymphocytes while there is little if any detectable AhR or ARNT protein or CYP1A1 mRNA or catalytic activity. Mitogen stimulation of human lymphocytes increased expression of all endpoints in a time-dependent manner. CYP1A1 and CYP1B1 mRNA levels and CYP1A1 enzyme activity were increased by *in vitro* treatment with TCDD. The range of interindividual variation in expression of these endpoints was 8-fold (TCDD-induced CYP1A1 activity) to 43-fold (resting AhR mRNA).

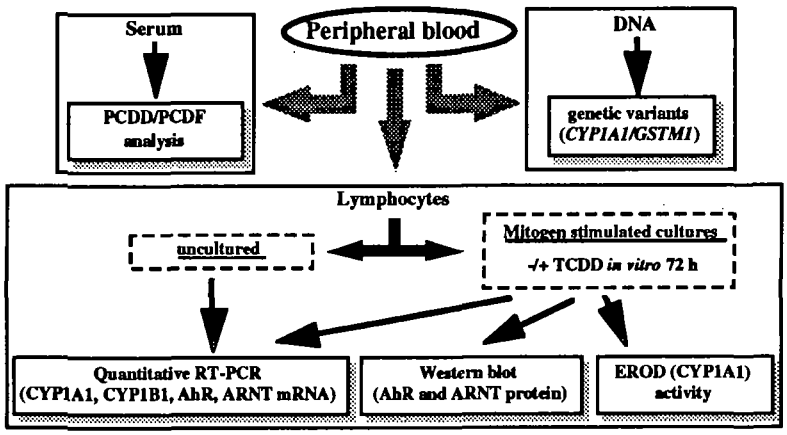

Fig. 1. Experimental approach for the analysis of dioxin biomarkers in human peripheral blood.

Dioxin biomarker studies in occupationally exposed individuals. The workers under study were employed at a chemical plant in Hamburg-Moorfleet, Germany operated by Boehringer-Ingelheim from 1951-1984 that produced organochlorine herbicides and pesticides (including 2,4,5-trichlorophenoxyacetic acid) and opioids. TCDD contamination at this plant was confirmed in 1984 by analysis of precursor materials, products, waste, and soil [15]. Mortality from all cancers [15,16] and ischemic heart disease[16] is elevated in a cohort of workers employed at this plant for at least 3 months from 1952-1984. Peripheral blood for lymphocyte isolation and serum dioxin analysis was obtained in 1992 for 110 individuals from this cohort who have undergone extensive medical examination (University of Mainz). Included in this group are individuals with expected low, medium, and high dioxin exposure. The range of serum TCDD concentration in the 110 individuals under study was 0-600 ppt lipid and the total TEQ of all PCDD/PCDF/PCB congeners ranged from 1-915 ppt lipid (data not shown). PCDD and PCDF exposure assessment of other subsamples of the Boehringer cohort appear elsewhere [16-18]. The skin disorder chloracne is considered a high dose response to dioxin exposure. Of the individuals in this study, 34/110, but not all of those highly exposed, developed chloracne at some point during their employment.

CYP1A1 enzyme activity in German chemical plant workers. CYP1A1 enzyme activity measured as TCDD-inducible EROD activity in cultured lymphocytes from 34 workers is shown in Fig. 2. The total range of variation in EROD activity in this subgroup was 15-fold. Approximately 50% (18/34) of the values lie within a narrow range (2-3.5 pmol/min/mg). CYP1A1 activity and dioxin levels in workers previously diagnosed with chloracne and in workers who did not develop chloracne is shown in Table 3. Serum TCDD, serum total dioxin TEQ levels, and lymphocyte CYP1A1 activity were not significantly different between the two groups.

Table 1. Expression of dioxin-related endpoints in human peripheral blood lymphocytes.

endpoint	uncultured	cultured with mitogen	cultured with mitogen+TCDD
AhR mRNA[a]	4-184	27-274	31-398
ARNT mRNA[a]	1-14	15-68	19-39
CYP1A1 mRNA[a]	<0.05	2-12	38-151
CYP1B1 mRNA[a]	0.2-4	2-13	34-104
AhR/ARNT protein	not detected	↑↑↑	↑↑↑
CYP1A1 activity[b]	not detected	0.04-0.76	0.89-6.49

a Values are expressed as the minimum-maximum molecules of mRNA ($x10^{-5}$)/ug total RNA.
b CYP1A1 activity is measured as EROD activity (pmol/min/mg protein).

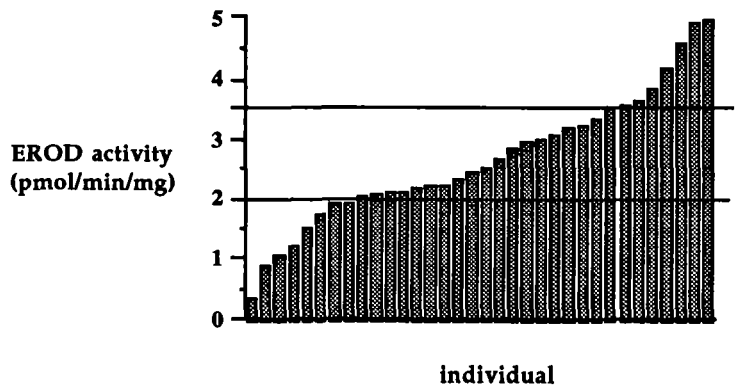

Fig. 2. CYP1A1 enzyme activity in individuals occupationally exposed to dioxins. The values represent net EROD activity (TCDD-treated minus control) in mitogen-stimulated lymphocytes from 34 workers.

Table 2. CYP1A1 activity and serum dioxin levels in German chemical plant workers previously diagnosed with chloracne.

	chloracne	no chloracne
n[a]	16	15
TCDD[b] (ppt)	32.1 (3.4-382.2)	11.5 (0-236.2)
TEQ[b,c] (ppt)	124.1 (22.7-914.7)	64.5 (16.6-664.3)
CYP1A1 activity[d]	2.81 (1.14)[e]	2.32 (0.89)

a The chloracne status of 3 individuals in the subgroup of 34 is unknown.

b TCDD and TEQ are the median (minimum-maximum) serum values expressed as ppt (pg/g) lipid.

c TEQ is the total dioxin TEQs for all PCDD/PCDF/PCB congeners analyzed.

d CYP1A1 activity is expressed as the mean (SD) net (TCDD induced-uninduced) EROD activity (pmol/min/mg) in lymphocyte cultures.

e n=18 for CYP1A1 activity in the chloracne group.

Conclusions

Quantitative analysis of AhR and dioxin-inducible gene expression in human peripheral blood lymphocytes is a suitable model system for investigating interindividual variation in markers of exposure and response to dioxin. In this preliminary study, dioxin responsiveness (measured as TCDD-inducible CYP1A1 activity) varied 15-fold while exposure (serum TEQ levels) varied 55-fold. Quantitative estimates of these two parameters, exposure and response, would lead to a biologically-based prediction of the range of expected risk. Thus, in a high exposure occupational scenario, the range of exposure rather than the range of biological response might be expected to contribute more to the range of expected risk. The magnitude of human variability is of great concern when dealing with extrapolation of animal data to human exposure. Although much is known regarding the magnitude and range of dioxin exposure in humans, the lack of knowledge regarding potential human response to dioxin creates one source of uncertainty when attempting to assess risks associated with dioxin exposure. Current safety factor approaches for human risk assessment use an uncertainty factor of ten to account for human interindividual variation. Such factors may not be adequate given the variability in human exposure and response. Mechanistically based biochemical markers of dioxin response provide a biologically relevant measure of exposure and may predict/identify highly responsive individuals at increased risk for dioxin-associated disease.

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