The Ah Receptor Is Not Involved in 2,3,7,8-Tetrachlorodibenzo-p-dioxin-mediated Apoptosis in Human Leukemic T Cell Lines*

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2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a common environmental pollutant causing public concern. Its toxic effects include disruption of the immune, endocrine, and reproductive systems, impairment of fetal development, carcinogenicity, and lethality in rodents. Here, we report that TCDD induces apoptosis in two cultured human leukemic lymphoblastic T cell lines. This cell death was found not to be dependent on an aryl hydrocarbon receptor and to be inhibited by the inhibitor of tyrosine kinases and caspases. Apoptosis-linked c-Jun N-terminal kinase is rapidly activated in these cells by the treatment with TCDD. A dominant-negative mutant of c-Jun N-terminal kinase prevented cell death in the treatment with TCDD. Furthermore, TCDD decreases the Bcl-2 protein level in these cell lines. These findings will help in the understanding of the molecular mechanism underlying TCDD-mediated immunotoxicity.

** Materials and Methods

Cell Culture and Apoptosis Assay—Cells were grown in RPMI 1640 medium containing 10% fetal calf serum, 10 IU/ml penicillin, and 0.1% streptomycin at 37 °C, in 5% CO₂. Treatment of cells with TCDD was conducted as follows. Exponentially growing cells in RPMI 1640 medium containing 10% fetal calf serum were collected, and fresh medium was added. In this condition, cells were grown for another 4-6 h at 37 °C. Then, cells were collected and washed once with phosphate-buffered saline (PBS). Cells were incubated at a density of 2.0 × 10⁵ cells/ml in serum-free RPMI 1640 medium either in the presence of TCDD or in an equal volume of solvent Me₃SO (concentration never exceeded the 0.1% level). Cells were harvested at different time points for the apoptosis assay.

Preparation of Cytoplasmic DNA—Cells (1.0 × 10⁶) were collected and suspended in 500 µl of 100 mM TrisCl, pH 8.0, containing 0.5 mg/ml proteinase K, 0.2 mM NaCl, 0.2% SDS, and 0.5 mM EDTA, then incubated overnight at 37 °C. Soluble cytoplasmic DNA was collected by centrifugation at 27,000 × g for 75 min at 4 °C, intact chromosomal DNA remaining in the pellet. The supernatant containing soluble apoptotic DNA was pelleted using isopropanol. The pelleted DNA was resuspended in 25 µl of TE buffer (10 mM TrisCl, pH 7.5, 1 mM EDTA) containing 0.1 mM MgCl₂, then incubated at 37 °C for 2 h. The DNA (5.0 µl) was applied to 20% agarose gel electrophoresis and visualized by ethidium bromide staining.

Cytotoxicity Assays—Cells were collected (0.5 × 10⁶ to 1.0 × 10⁶ cells) by centrifugation at 300 × g for 10 min, then washed once with PBS. Cells were resuspended in 50 µl of 3% paraformaldehyde in PBS, then incubated for 10 min at room temperature. Fixation solution was removed by centrifugation and washing with PBS. Fixed cells were resuspended in 15 µl of bis-benzimide (Hoechst 33258) solution (16 µg/ml in PBS), then incubated for 15 min at room temperature. Stained
Ah Receptor-independent Apoptosis by TCDD

Ah receptor-independent apoptosis (11) was not observed in L-MAT and Jurkat cells. In one experiment, L-MAT cells with 20 nM TCDD also resulted in an apoptotic DNA ladder consisting of 180-base pair fragments in the agarose gel (Fig. 1A). As a control, DNA was prepared from an equal volume of solvent (dimethyl sulfoxide)-treated cells. A DNA ladder was routinely observed within 4 h of treatment with TCDD. We extracted only the degraded DNA, not the intact chromosomal DNA, from apoptotic as well as nonapoptotic cells. This DNA extraction method simplified sample loading onto the gel. Sample loading was optimized as follows. Samples of soluble apoptotic DNA obtained from the same number of cells were dissolved in equal volumes of TE buffer, and the same volume of sample was applied to each lane. This TCDD-mediated apoptosis was not restricted to L-MAT cells; another lymphoblastic T cell line, Jurkat, also showed the characteristics of apoptosis when treated with TCDD (Fig. 1A).

Morphological Alterations in Chromatin—Incubation of L-MAT cells with 20 nM TCDD also resulted in the appearance of morphological changes characteristic of apoptosis upon staining with the DNA-specific fluorochrome bis-benzimide (Fig. 1B). These changes include condensation of chromatin, its compaction along the periphery of the nucleus, and segmentation of the nucleus. The early stages of the cytoplasmic and nuclear morphological typical of apoptosis were revealed by electron microscopy (Fig. 1C).

Kinetics of TCDD-mediated Apoptosis—The time course of changes in L-MAT cell viability was measured by means of a trypan blue exclusion assay. An increase in the number of nonviable cells became apparent at 2–4 h after the addition of TCDD to the culture medium and, at 8 h, nearly 90% of the cells exhibited a loss of viability (Fig. 1D). This apoptotic effect was observed with doses of TCDD as low as 1 nM, and a plateau of nearly 90% cell death at 8 h was observed with doses of 20 nM or more TCDD (Fig. 1E).

Specificity of Action of TCDD—To examine the specificity of TCDD-mediated apoptosis, we tested other Ah receptor ligands for the induction of apoptosis in L-MAT cells. A close structural analog, 2,3,7,8-tetrachlorodibenzofuran (TCDF), failed to induce apoptosis at a similar concentration (Fig. 2A). Another Ah receptor ligand, β-naphthoflavone, also failed to induce apoptosis even at a 1000-fold molar excess (20 μM) in this cell line (Fig. 2A). These results demonstrate both the specificity of action of TCDD and the usefulness of this system as an in vitro model for assaying TCDD-mediated toxicity.

Effects of Cycloheximide (CHX) on TCDD-induced Apoptosis—Some of the initial evidence suggested that apoptosis is caused by an active process, and that new protein synthesis is required for apoptosis (13). However, it was subsequently shown that a macromolecular synthesis inhibitor failed to block apoptosis in many systems and, indeed, could even induce this process in many situations (14). To determine whether macromolecular synthesis is essential for TCDD-induced cell death, L-MAT cells were pretreated for 2 h with CHX or actinomycin D, to block new protein synthesis. Then, 2 h later, TCDD was added to the cells. Actinomycin D itself proved to be cytotoxic to L-MAT cells (data not shown). Treatment with 20 nM TCDD and CHX (10 μM) failed to inhibit apoptosis (Fig. 2B). However, CHX was found to increase the sensitivity of the cells to TCDD, although CHX alone did not have any effect on this cell line (Fig. 2B). We also confirmed that, in our hands dexamethasone-induced cell death is inhibited by CHX (data not shown).

Ah Receptor Is Not Present in Cell Lines—We thought it possible that CYP1A1-mediated oxygen radicals might be the major cause of the apoptosis induced by TCDD in these cell lines. To test this possibility, we checked the inducibility of CYP1A1 in L-MAT and Jurkat cells. In fact, RT-PCR revealed that CYP1A1 is not inducible in either of these two cell lines by
2 nM TCDD, although we could routinely use this concentration of TCDD to induce CYP1A1 in HepG2 cells (Fig. 3A). It could be argued that the serum-free culture conditions used for the apoptosis assay had already induced CYP1A1. In an attempt to rule out this possibility, we prepared RNA from dying cells (20 nM TCDD-treated apoptotic cells), then performed RT-PCR. As expected, CYP1A1 was not induced during the apoptosis (Fig. 3B). These results clearly indicate that CYP1A1-mediated oxygen radicals are not the cause of the apoptosis produced by TCDD.

**TCDD Mediates Apoptosis in the Absence of the Ah Receptor**—The finding that inhibition of protein synthesis failed to block the induction of apoptosis by TCDD in L-MAT cells (Fig. 2) raised the possibility that direct transcriptional activation of death gene(s) by the Ah receptor is not required for the induction of apoptosis by TCDD. Like glucocorticoid and retinoic acid receptors (15), the Ah receptor might cause transcriptional repression of some survival genes in TCDD-mediated apoptosis. To test this hypothesis, we first examined the expression level of the Ah receptor in our cell lines. In fact, using RT-PCR, we could not detect any mRNA for the Ah receptor in either the L-MAT or Jurkat cell line (Fig. 3A). In contrast, HepG2 cells showed the presence of Ah receptor mRNA in both TCDD-treated and nontreated samples (Fig. 3A). This result was verified by three independent experiments, with the RT-PCR standardized using different numbers of cycles (25–35 cycles) and using different amounts of mRNA (range, 0.5 to 5.0 μg).

The results of RT-PCR using mRNA from dying cells were essentially the same as those described above (Fig. 3B).

**Protein-tyrosine Kinase and Caspases Inhibitors Completely Block TCDD-mediated Apoptosis**—Next, we tried to determine which signaling events that might mediate the apoptosis caused by TCDD in these cell lines. Besides transforming the Ah receptor, TCDD activates protein-tyrosine kinases in responsive cells (16, 17). Moreover, genistein, a selective protein-tyrosine kinase inhibitor, has been found to block apoptosis (18, 19). To determine whether protein-tyrosine kinase is involved in TCDD-mediated apoptosis, we used genistein to try to block the apoptosis produced by TCDD. A range of different concentrations of genistein was used (10–50 μg/ml) in L-MAT cells and, as expected, 50 μg/ml genistein completely blocked the induction of cell death by TCDD (Figs. 4 and 5). Genistein alone had no effect in these cell lines (data not shown).

In mammalian cells, caspases (formerly known as interleukin-1β-converting enzyme (ICE) or ICE-related proteases) have been implicated in apoptosis (20, 21). We therefore investigated whether proteolytic activity of caspases might also be involved in TCDD-mediated apoptosis. In L-MAT cells, the apoptosis triggered by TCDD was strongly inhibited by the caspases inhibitor, carbobenzoxyl-L-aspartyl-α-[2,6-dichlorobenzoyl]oxy] methane (Z-Asp-CH₂-DCB) (22) (Figs. 4 and 5). We then tested other protease inhibitors: (i) the calpain inhibitor, leupeptin; (ii) the classical serine protease inhibitor, phenylmethylsulfonyl fluoride; and (iii) the cysteine protease inhibitor,
antipain. However, all three failed to inhibit TCDD-mediated cell death (data not shown).

**JNK Is Activated by TCDD in L-MAT and Jurkat Cells**—In neuronal (23) and hematopoietic (24) model systems, apoptosis induced by stress was linked to the sustained activation of JNK. Therefore, JNK activity was measured upon TCDD treatment in L-MAT cells. Induction of JNK activity was observed within 30 min upon TCDD treatment in L-MAT cells (Fig. 6). The peak activity of JNK was observed 90 min after TCDD treatment, and similar results were obtained in Jurkat cells (data not shown). These results revealed that TCDD-mediated apoptotic signals strongly induced JNK activity, which coincides with cell death.

**Dominant-negative Mutant of JNK Inhibits TCDD-mediated Cell Death**—To determine the role of JNK in TCDD-mediated cell death in these cells, we tested the effect of interfering with JNK function by transient expression of a dominant-negative mutant of JNK (APF) in L-MAT cells. To identify the transfected cells, cells were transfected with β-galactosidase-expressing vector, pCAG, with or without the dominant-negative mutant of JNK. The cells were stained with anti-β-galactosidase antibody and fluorescein isothiocyanate-conjugated secondary antibody. For the identification of the apoptotic cells, in which the DNA was degraded, nuclei were stained with Hoechst dye 33258. In β-galactosidase-expressing cells, the dominant-negative JNK should be expressed, and normal JNK should be inhibited. Thus, the survival rate of transfected cells was determined as the percentage of apoptotic cells in β-galactosidase expressing cells in TCDD and Me2SO-treated cells. Expression of dominant-negative mutant of JNK blocked TCDD-mediated apoptosis (Fig. 7), suggests a direct role of JNK signal transduction pathway in the TCDD-mediated cell death in these cell lines.

**TCDD Down-regulates BCL-2 Expression in Cell Lines**—We next asked whether TCDD-mediated apoptosis in these cells is associated with modulation of Bcl-2 protein expression. Using Western blot analysis, we found that Bcl-2 protein levels decreased as early as 2 h after onset of culture with TCDD and then declined further by 6 h of culture (Fig. 8).

**DISCUSSION**

For a long time it has been thought that TCDD is not toxic to cultured cells (25, 26). However, the data reported in this study demonstrate for the first time that TCDD does cause cell death in cultured lymphoblastic T cell lines, and that this cell death exhibits distinct apoptotic features. The classically accepted morphological features of apoptosis including nucleoplasmic and cytoplasmic condensation, the formation of apoptotic bodies, membrane blebs, and loss of cell volume, were seen following TCDD treatment. These
substrate phosphorylation was detected by autoradiography. The trophoresed on SDS-polyacrylamide gel electrophoresis, 10% gel, and indicates the phosphorylated glutathione (amino acids 1–79).

Inhibition of TCDD-mediated apoptosis by genistein (Gen) and by Z-Asp-CH2-DCB (Z-Asp). Cells were treated with different concentrations of either genistein (10–60 \( \mu \)g/ml) or Z-Asp (10–60 \( \mu \)g/ml). Cell viability was assessed by the trypan blue exclusion method.

extensive cyto-architectural modifications, in conjunction with the induction of internucleosomal DNA fragmentation, a hallmark of apoptosis, established that TCDD is indeed toxic to at least these cultured cells and that the cell death mediated by TCDD is classical apoptosis. Since caspases are involved in many kinds of apoptosis in higher eukaryotes (21), the inhibition of TCDD-mediated cell death by selective caspase inhibitors also suggested that this cell death is an example of apoptosis. Possibly, the apoptosis mediated by TCDD may play an important role in the immunotoxicity of this agent.

The induction of the xenobiotic metabolic enzyme, CYP1A1, by TCDD in vitro and in vivo has been well characterized in many experimental systems (27). Recently, it has been found that induction of CYP1A1 in the mouse hepatoma cell line, Hepa-1, produces oxygen radicals which then cause oxidative DNA damage (28). In fact, oxygen radicals are involved in the apoptosis induced by many kinds of extracellular stimuli (29). However, we found that CYP1A1 is not inducible in these cell lines by TCDD, and consequently we can say that CYP1A1-mediated formation of oxygen radicals is not involved in the apoptosis studied here. However, we cannot rule out the possibility that oxygen radicals are generated from other sources by TCDD in these cell lines; such oxygen radicals could be involved in this apoptosis.

That the Ah receptor is not required for the TCDD-mediated induction of apoptosis in these cell lines was established by the following findings: 1) ligands other than TCDD did not cause apoptosis at doses sufficient to activate the Ah receptor (30–32), 2) CYP1A1 was not inducible in these cell lines (i.e. a functional Ah receptor is not present in these cell lines), and 3) the mRNA for the Ah receptor was not present in either of the two cell lines tested. We stably transformed the human Ah receptor gene in both L-MAT and Jurkat cells and checked the kinetics of the TCDD-mediated apoptosis. As we expected, there were no differences between the Ah receptor-transformed and nontransformed cells with respect to the kinetics of TCDD-mediated apoptosis (data not shown). These findings are well supported by other studies. For example, in Hepa-1 cells, TCDD has been found to induce c-Fos, c-Jun, JunB, and JunD mRNA (33). This induction also occurred in Ah receptor-less and Ah receptor nuclear translocator mutant cell lines (c2 mutant cells and c4 mutant cells, respectively). This suggests that an Ah receptor-TCDD interaction may not be involved in some of the actions of TCDD (34). The Ah receptor is present in all the mammalian species so far checked, but the binding affinity of TCDD for the Ah receptor differs from one species to another (35). Moreover, the actual binding affinity does not always correlate with the TCDD-mediated toxicity in a given species (1). Indeed, only two strains of mice (C57BL/6J and DBA/2) have shown a good relationship between sensitivity to TCDD (in terms of induced toxicity) and Ah receptor binding affinity (36). Taken together, this suggests that besides the TCDD-Ah receptor interaction, additional TCDD-activated signal-transduction pathway(s) may also play an important role in the toxicity of this agent.

One recent report suggested that apoptotic machinery is constitutively present in virtually all cell types in mammals, and that on-going transcription is required in some cases for the activation of signal transduction pathways to occur (37). Our results suggest that, in the present lymphoblastic T cell lines, TCDD probably activates only the preexisting death pathway, new protein synthesis not being required. However, we cannot rule out the other possibility purely on the basis of the effects of CHX treatment on these cells. CHX has been
proven to be an activator of c-Jun N-terminal kinases or stress-activated protein kinases, which are directly involved in stress-activated apoptosis in T cell lines (24). The present finding that CHX did not inhibit apoptosis is in contrast to an earlier report concerning TCDD-mediated apoptosis in a rat thymocyte culture (3). The most likely explanation for the discrepancy is that we used human cultured lymphoblastic T cell lines, whereas the previous study used immature rat thymocytes.

In keratinocytes, genistein, a selective inhibitor of protein tyrosine kinases, completely blocks the induction of CYP1A1 by TCDD (38), and induction of transforming growth-factor-β by TCDD is also inhibited by genistein (39). In the present study, genistein completely blocked the apoptosis mediated by TCDD in the absence of the Ah receptor. Depending on the cellular context, tyrosine kinases transmit either cell survival or cell death signals. Long term exposure of the cell to protein-tyrosine kinase inhibitors can itself cause apoptosis in some systems (40); however, we used genistein for a relatively short period of time. Our results suggest that, at least in the present cell lines, activation of protein-tyrosine kinase is essential for TCDD-mediated apoptosis, and perhaps for some of the other toxic effects of the TCDD, notably its immunotoxicity.

The connections between TCDD signaling and the apoptosis were explored in this study. This motivated the examination of the role of the stress-response signal transduction pathway in the TCDD-mediated apoptosis in these cell lines. Stress-activated protein kinase JNK is rapidly activated in these cell lines by TCDD. The JNK pathway is well known to respond to extracellular stimuli that induce apoptosis, such as ceramide, granzyme B, interleukin-1, UV, and γ-radiation (23, 24, 41–43). Latter, it has been found that JNK mediated this apoptotic process. Sustained JNK activity is up-regulated severalfold in these cell lines by TCDD. This result clearly demonstrates that TCDD treatment regulates the well characterized stress response pathway. The mechanism by which TCDD treatment triggers the sustained JNK activation remains to be elucidated. A dominant-negative mutant of JNK blocked the TCDD-mediated cellular death in these cells. This further supports the notion of the role of a JNK signal transduction pathway in the TCDD-mediated apoptosis.

The central role of Bcl-2 in apoptosis was well established by numerous studies (44, 45). Posttranslational modification of Bcl-2 has a major impact in apoptosis. Activation of the Bcl-2 by proteases degradation has been implicated as a mechanism by which the death signal can neutralize the anti-apoptotic function of Bcl-2 and enhance cell death (46). We found evidence that apoptosis mediated by TCDD is preceded by down-regulation of Bcl-2 protein in these cell lines. These findings suggest that down-regulation of Bcl-2 protein is one of the factors playing an important role in the pathway of TCDD-induced apoptosis of these cells.

In summary, the results of the present study will help in the understanding of the molecular mechanism by which TCDD exerts its toxic actions in humans. It is tempting to speculate that, in mammalian species, the immunotoxicity of TCDD is partly due to a direct action on signal transduction pathways (i.e., an action independent of the Ah receptor), and partly to an action that does depend on the formation of Ah receptor-TCDD complexes. A better understanding of the molecular mechanisms underlying TCDD-mediated toxicity will help in assessing the risk to humans of exposure to TCDD.