MECHANISM OF TCDD-MEDIATED APOPTOSIS IN HUMAN T-LYMPHOBLASTIC CELL LINES

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Introduction:

The adverse biological effects of TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) seen in experimental animals include immune, reproductive, and developmental toxicity, carcinogenicity, wasting syndrome, chloracne, and lethality (1). While the immunotoxic effects of TCDD have been well characterized in the rodent model, little data is available for humans. The thymic atrophy caused by TCDD in rodents is mediated by a selective killing of immature thymocytes, although the exact mechanism remains unknown. Immature thymocytes treated with TCDD (*in vitro)* have been reported to die due to apoptosis in the rat (2) and in the mouse (3), although other studies have produced contradictory results (4). While it is clear that TCDD is highly toxic to mammals, the cellular mechanisms by which TCDD exerts its toxic effects are obscure. We now describe a cell culture system derived from human T-lymphomas, L-MAT (5), that provides unique advantages for studying the molecular mechanisms underlying the action of TCDD.

Materials and Methods:

Cell culture and apoptosis assay: Cells were grown in RPMI 1640 medium containing Treatment of cells with TCDD was carried out as follows: exponentially growing cells in RPMI 1640 medium containing 10% fetal calf serum (FCS) 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 PBS . Cells were incubated at a density of 2.0×10^5 cells/ml in serum-free RPMI 1640 medium either in the presence of TCDD or in an equal volume of solvent DMSO (concentration never exceeded the 0.1% level). Cells were harvested at different time points for the apoptosis assay.

Preparation of RNA and RT-PCR: Total RNA was prepared from the cells by the usual method and the prepared RNA (0.5-5.0 µg) was reverse-transcribed (to synthesize cDNA) by means of AMV reverse transcriptase (Life Sciences) using a random hexamer. Primer sequences were from published sources, and were as follows.

AHR: TTGGCTTTGTTTGCGATAGCT and CTGCATGTGTCTGATGTCTTC;
Cytochrome P450 1A1 (CYP1A1): TCCATCAGCATCTATGTGGC
and TTCATCCCTATTCTTCGCTAC.

PCR reactions were carried out in a final volume of 20.0 µl containing 1/10 volume of the RT sample, 2.0 µl of 10x Taq buffer, 500 µM of each dNTP in the presence of 1.0 µM of each primer, and 2.5 units of Taq DNA polymerase (Gibco-BRL). The PCR products (5.0 µl) were then subjected to 1.5% agarose gel electrophoresis and stained with ethidium bromide.

In vitro (solid phase) JNK assay: The solid phase JNK assay was carried out by using a GST-cJun (1-79) fusion protein coupled to glutathione beads as a substrate according to published report (6).

Transient transfection assay: L-MAT cells were cotransfected with pCAG plasmid expressing β-galactosidase and dominant-negative mutant of JNK1, pcDNA3-Flag-JNK1(APF) (a gift from Dr. Roger J. Davies) (7), by the lipofectamine method according to the manufacturer's (Gibco-BRL) instructions.

Immunoblot: Cells were treated with 20 nM TCDD for different time period and cell extracts were prepared as described in JNK assay. Protein (100 µg) was electrophoresed in 13.5 % SDS-PAGE and transferred on to the nitro cellulose membrane. The membrane was immuno stained with anti-Bcl-2 monoclonal antibody, clone Bcl-2-100 (Sigma) and was developed with enhanced chemiluminescent kit (Amersham International PLC).

Results and Discussion

Induction of apoptosis by TCDD: Treatment of L-MAT cells with 20 nM TCDD in serum-free culture medium resulted in an apoptotic DNA ladder consisting of 180 bp fragments in the agarose gel. This TCDD-mediated apoptosis was not restricted to L-MAT cells; another T-lymphoblastic cell line, Jurkat, also showed the characteristics of apoptosis when treated with TCDD.

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. 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 morphology typical of apoptosis were revealed by electron micrography. These 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.

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 non-viable 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.

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. Another Ah receptor ligand, ß-naphthoflavone (ß-NF) also failed to induce apoptosis even at a 1000-fold molar excess (20 µM) in this cell line. 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.

Ah receptor is not present in these cell lines : 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. In contrast, HepG2 cells showed the presence of Ah receptor mRNA in both TCDD-treated and non-treated samples. This result was verified by three independent experiments, with the RT-PCR standardized using different numbers of cycles (25 cycles to 35 cycles) and using different amounts of mRNA (range, 0.5-5.0 μg). The results of RT-PCR using mRNA from dying cells were the essentially the same as those described above.

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; (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. Taken together, all 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.

PTK and caspases inhibitors completely block TCDD-mediated apoptosis:

To determine whether PTK 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. Genistein alone had no effect in these cell lines. Our results suggest that, at least in the present cell lines, activation of PTK is essential for TCDD-mediated apoptosis, and perhaps for some of the other toxic effects of the TCDD, notably its immunotoxicity. We 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) (8). We then tested other protease inhibitors: (i) the calpain inhibitor, leupeptin, (ii) the classical serine protease inhibitor, phenyl methyl sulfonyl fluoride (PMSF), and (iii) the cysteine protease inhibitor, antipain. However, all three failed to inhibit TCDD-mediated cell death. Since caspases are involved in many kind of apoptosis in higher eukaryotes (9). the inhibition of TCDD-mediated cell death by a selective caspases inhibitor 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.

JNK is activated by TCDD in L-MAT and Jurkat cells: In a neuronal model systems (10), 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. The peak activity of JNK was observed 90 min after TCDD treatment, and similar results were obtained in Jurkat cells. These results revealed that TCDD-mediated apoptotic signals strongly induced JNK activity, which coincides with cell death.

Dominant-negative mutant of JNK inhibits the 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 dominant-negative mutant of JNK. The cells were stained with anti-□-galactosidase antibody and FITC-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 DMSO treated cells. Expression of dominant-negative mutant of JNK blocked TCDD-mediated apoptosis, suggests a direct role of JNK signal transduction pathway in the TCDD-mediated cell death in these cell lines.

Sustained JNK activity is upregulated several fold in these cell lines by TCDD. This results clearly demonstrate that TCDD treatment regulates the well characterized stress response pathway. These notion further support the role of JNK signal transduction pathway in the TCDD-mediated apoptosis.

TCDD down-Regulates BCL-2 Expression in these 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 hr after onset of culture with TCDD and then declined further by 6 hr of culture.

Activation of the Bcl-2 by proteases degradation has been implicated as a mechanism by which death signal can neutralize the anti-apoptotic function of Bcl-2 and enhance cell death (11). We find 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.

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