Water-soluble 2,3,7,8-tetrachlorodibenzo-*p*-dioxin complex with human α-fetoprotein: properties, toxicity in vivo and antitumor activity in vitro

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Abstract The conditions for the formation of a non-covalent complex between 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and the human transport fetal protein, α-fetoprotein (AFP), have been studied. TCDD has been shown to form a stable complex with AFP in a 2:1 (TCDD:AFP) ratio. The apparent solubility of TCDD in water increases 10^5-fold after complex formation. The toxicity of the TCDD:AFP complex injected into mice by the intravenous route is comparable with that of free TCDD administered in oil solution per os. The complex manifests very much higher toxicity (200–1400 times) against human tumor cells (CEM, MCF-7, HepG2) in vitro and surpasses TCDD in selectivity. AFP may facilitate TCDD transport in embryonic tissues and enhance its embryotoxic and teratogenic effects.

Key words: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; α-Fetoprotein; Complex; Solubility; Toxicity; Embryotoxicity

1. Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), commonly referred to as dioxin, belongs to the class of the most hazardous environmental contaminants. It is formed as a by-product in many technological processes and manifests high environmental stability and a broad spectrum of biological activities. Polychlorinated dioxins and many related compounds (polychlorinated dibenzofurans, biphenyls and other aromatic hydrocarbons) are distinguished for high toxicity and carcinogenicity [1–5]. Besides, they promote carcinogenesis and are endowed with immunotoxic, embryotoxic, teratogenic and neurotoxic activities [6–8]. These compounds can also produce atrophic changes in lymphoid organs (thymus, spleen, lymph nodes) and influence the function of adrenal, thyroid, mammary and sex glands [6,8]. The characteristic property of TCDD which sets it apart from the majority of toxic compounds is that the life span of experimental animals in acute toxicity studies only weakly depends on its dose; animal death occurs as a result of the so-called wasting syndrome manifested as progressive and irreversible loss of body weight [8]. Other distinctive features of TCDD are significant interspecific differences in acute toxic effects [9–11] and high embryotoxicity [12–14].

The aim of the present work was to study the interaction of TCDD with the fetal transport protein human α-fetoprotein (AFP), and to examine the biological properties of the transport form of TCDD after its binding to AFP in experiments with mice and human tumor cells.

2. Materials and methods

TCDD (Cambridge Isotopes Lab, USA) was dissolved in HPLC-grade 1,4-dioxane (Fluka, Switzerland) at 0.8–0.9 mg/ml concentration. The solution was stored in argon atmosphere at 4°C. Human AFP was isolated as described previously [15].

TCDD dissolved in 1,4-dioxane was added slowly upon gentle shaking to a solution of AFP (protein concentration was 1.5–2.0 mg/ml) in phosphate-buffered saline pH 7.4 (PBS) to a final 1,4-dioxane concentration of not more than 5% (v/v). The reaction mixture was incubated for 1–6 h at 30–37°C. After incubation the AFP:TCDD complex was separated from low molecular weight components on a Sephadex G-25sf column (Pharmacia, Sweden) equilibrated with PBS containing 10% acetonitrile (v/v) (HPLC-grade, Fluka, Switzerland). The fractions of the target complex were combined and stored at 4°C until analysis (as a rule, for no longer than 48 h). For long-term storage dry mannitol (up to 10% w/v) was added to AFP:TCDD, after which the solution was passed through a micro-filter (0.22 μm) under sterile conditions, poured out into glass tubes, frozen and lyophilized. After freeze-drying the tubes were filled with argon, soldered up and stored at −70°C.

TCDD was quantitatively assayed by reverse phase HPLC on a 'GOLD' liquid chromatograph (Beckman, USA). The chromatographic system consisted of a gradient pump M-126 (Beckman, USA), a loop injector M-7125 (loop volume 500 μl) (Rheodyne, USA), an 0.46×7.5 cm Ultrasphere Octyl column (3 μ) and a flow spectrophotometer M-167 (Beckman, USA). The separation was performed at a flow rate of 1.5 ml/min as linear gradient elution of component B (65–85%) (A = 0.1 M ammonium acetate, pH 6.0, 10% acetonitrile (v/v); B = 50:50 acetonitrile:isopropanol) for 10 min. The TCDD:AFP complex was applied onto the column in a volume of 5–500 μl. The assay was performed by the external standard method using a TCDD solution in 1,4-dioxane (0.1–0.2 mg/ml). Peak area measurements and treatment of experimental results were performed using a chromatographic program 'GOLD Personal Chromatograph' (Beckman, USA). Mean values were obtained from three parallel concentration measurements in both sample and standard solutions. The experimental error was calculated by the least squares method.

Acute toxicity of the TCDD:AFP complex (0.05–2.0 mg/kg) was estimated in experiments with C57BL/6 mice of both sexes (18–20 g body weight). Control groups included 10 animals; each experimental group consisted of six animals. The solution of the TCDD:AFP complex in PBS was injected into the tail vein in the volume of 0.1–0.15 ml. The death of experimental animals was followed up to day 45 after the injection. LD_{50} was calculated by the probit method; mean values and standard deviation were calculated at $P = 0.05$.

The biological activity of the TCDD:AFP complex in vitro was tested in experiments with the following human tumor cell lines: CEM (T-cell lymphoma), MCF-7 (mammary carcinoma) and

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Abbreviations: AFP, human α-fetoprotein; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; FBS, fetal bovine serum; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline

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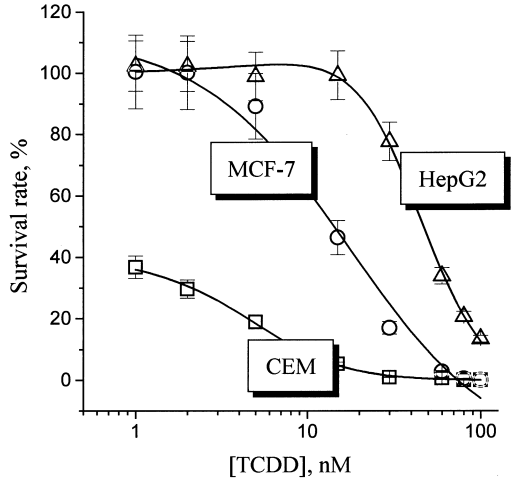

Fig. 1. Toxicity of the TCDD:AFP complex against human tumor cells in vitro.

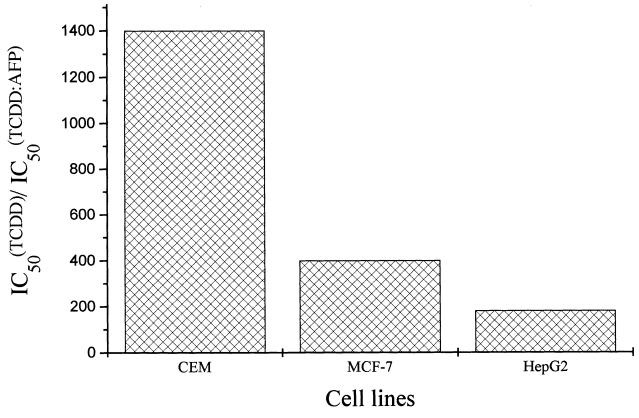

Fig. 2. Relative toxicity of the TCDD:AFP complex versus toxicity of free TCDD against human tumor cells.

HepG2 (hepatoma). CEM cells were cultured in RPMI 1640 medium (Sigma, USA) supplemented with 10% FBS (Gibco, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. MCF-7 and HepG2 cells were cultured under identical conditions except that DMEM medium (Sigma, USA) was used. Cytotoxic activities of TCDD and its complex with AFP were determined with the MTT test using the method of Mosmann [16]. TCDD was added to the cell medium in isopropanol; TCDD:AFP was added in PBS.

3. Results

Measurements of TCDD concentration in the complex prepared at various TCDD:AFP ratios (2–10 molar excess of TCDD) revealed that the AFP molecule has two binding sites for TCDD. After separation of the protein from an excess of non-bound ligand the TCDD concentration within the complex varied from 2 to 12 μg/ml. After concentration of the TCDD:AFP complex by ultrafiltration on a YM-10 membrane (Amicon, The Netherlands) samples with TCDD concentration of up to 25–30 μg/ml were obtained.

The toxicity of the TCDD:AFP complex and sex-related differences in the sensitivity of experimental animals to its effect were studied in C57BL/6 mice of both sexes. The experimental values of LD_{50} and average life spans of experimental mice are given in Table 1.

The cytotoxicity of the TCDD:AFP complex against all the cell lines tested in this study appeared to be much higher than that of free dioxin. Noteworthy, the difference in the sensitivity of cells of various lines to the effect of free TCDD was pronounced in a much lesser degree ($IC_{50} = 0.7–8$ μM). T-cell lymphoma (CEM) cells manifested the highest sensitivity to the effect of the TCDD:AFP complex ($IC_{50} < 0.50$ nM). Mammary adenocarcinoma cells (MCF-7) were more resistant ($IC_{50} = 18$ nM), while HepG2 hepatoma cells were the least sensitive under the given experimental conditions ($IC_{50} = 52$ nM).

4. Discussion

The data presented in Table 1 suggest that the TCDD:AFP complex administered as described above manifests higher toxicity in comparison with per os administration of free dioxin as a micellar suspension in a non-ionic detergent (1.18 and 1.62 mg/kg for female and male albino mice, respectively) but was less toxic in comparison with dioxin administered per os in vegetable oil (LD_{50} for albino mice was 0.046 mg/kg) [9]. The literature values of LD_{50} for male C57BL/6 mice to which TCDD was administered orally in vegetable oil are 114 [17] and 283 μg/kg [10]. The corresponding values for male C57BL/6 mice obtained in this study indicated that acute toxicity of TCDD injected intravenously as a AFP:TCDD complex differs significantly from the toxicity of free TCDD administered per os in oil solution. The observed differences in the LD_{50} values for male and female mice are significant at $P = 0.05$ which testifies to sex-related distinctions in the sensitivity of mice of this particular strain to the effect of the compound tested. The results of this study corroborate the literature data according to which female mice are more sensitive to the toxic effect of TCDD administered by alternative routes [9].

A graphic representation of the IC_{50} values of the TCDD:AFP complex for human tumor cell cultures is given in Fig. 1. Relative toxicities of dioxin and its complex with AFP for the same cell lines are displayed in Fig. 2. As seen, the difference in the sensitivity of human tumor cells to TCDD:AFP between the most and least sensitive lines is much higher than in the case of relative cytotoxicity (see Fig. 2).

Stipulating extremely low solubility of TCDD in water (~20 ng/l) [18], it may be concluded that the apparent solubility of dioxin increases more than 10^5-fold during the formation of the TCDD:AFP complex. By forming a complex with TCDD, AFP converts this compound into a 'transport' form and thus facilitates its delivery to various embryonic

Table 1
Toxicity for mice of the non-covalent TCDD:AFP complex administered by the intravenous route

Strain	Sex	LD_{50} (mg/kg)	Average life span (days)	Life span (days, min–max)
C57BL/6	Female	0.33 ± 0.08	17.0 ± 2.6	13–20
	Male	1.10 ± 0.17	13.9 ± 2.8	11–17

organs and tissues. Cytotoxicity data also provide evidence that the formation of the TCDD:AFP complex not only increases the solubility of this environmental toxicant in aqueous media but strongly enhances the selectivity of its biological effect on tumor cells from various human tissues. A comparison of cytotoxic effects of free dioxin and the TCDD:AFP complex for the most and least sensitive cell lines (CEM and HepG2, respectively) revealed that the IC_{50} values of dioxin for these cells differ between themselves by about one order of magnitude, while for the TCDD:AFP complex this difference is two orders of magnitude (8/0.7 = 11 and 52/0.5 = 104).

In this study we have developed a model of selective delivery of TCDD to target cells using AFP as a vector molecule. It is generally believed that in an embryonic organism AFP mainly fulfills the transport function by providing efficient exchange of free fatty acids, hormones and some other metabolites [19]. It has been found that apart from its high affinity for fatty acids, AFP has several binding sites (2–8) for steroid hormones, bilirubin and a great variety of medicinal drugs [20].

Taking into account the high efficiency of the transport function of AFP in vivo as well as the high hydrophobicity and some structural features of the TCDD molecule, it seemed appropriate to use AFP for targeted delivery of TCDD to human tumor cells in model systems and to elucidate the reasons for its high embryotoxicity.

This approach has made it possible to obtain a stable noncovalent complex in a ligand:protein molar ratio of 2:1. The formation of the TCDD:AFP complex caused a more than 10^5-fold increase in the apparent solubility of TCDD. It may thus be assumed that it is the formation of the TCDD complex with the major fetal transport protein, AFP, which markedly increases the efficiency of its targeted delivery to various embryonic organs and is responsible for its high embryotoxicity and teratogenicity.

The results of cytotoxicity studies of the TCDD:AFP complex in vitro as well as some evidence for high antiestrogenic activity of TCDD [21–24] point to the expediency of investigation of antitumor activity of the newly synthesized TCDD:AFP complex against hormone-dependent tumors in vivo.

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