THE INFLUENCE OF 2,3,7,8 – TETRACHLORODIBENZO-P-DIOXINE (TCDD) ON THE EROD-ACTIVITY, MITOGENIC RESPONSE, CELL VIABILITY, APOPTOSIS AND FAS-RECEPTOR EXPRESSION IN THE MITOGEN-STIMULATED HUMAN PERIPHERAL BLOOD LYMPHOCYTE CULTURES

Darya S. Sibiryak, Sergey V. Sibiryak, Nelly N. Kurchatova, Rina Sh. Yusopova, Zarema Amirova*, Edward A. Kruglov*

Department of Immunology, Russian Eye and Plastic Surgery Centre, 47, Koltsevaya St., 450040, Ufa, Bashkortostan, Russia. * Regional Ecological Scientific Centre. 147. October Av., 450075, Ufa, Bashkortostan, Russia

Introduction

The immunocompetent cells (T- and B-lymphocytes, monocytes/macrophages) are characterized by the expression of the wide spectrum of the constitutive and inducible CYP450 isoforms. There is an evidence that the CYP450 enzyme activity and the immune functions are interconnected. The immune activation caused the depression of the CYP450 enzyme activity in liver and other tissues. This effect is mediated via cytokine-induced inhibition of the different CYP450 proteins synthesis. The inhibitory effect is described for the α–, β–, γ - interferones, interleukins 1, 2, 6, TNF, TGFβ[1,2,3]. On the other hand the CYP450-inducers such as TCDD and related compounds caused marked immune abnormalities in the in vitro and in vivo model systems and in the exposured humans. There are reduced antibody production, the abnormal T- and B-cell maturation and differentiation, the abnormal mitogenic response and activation-assotiated surface antigens expression, stimulation of reactive oxygen species generation and proinfllammatory cytokines production[4,5,6]. The Ah-receptor mediated induction of the CYP1A1 is one of the most sensitive responses assotiated with the exposure to 2,3,7,8-TCDD and other polycyclic aromatic hydrocarbons (PAH) in lymphocytes and other tissues. This effect is considered as the main mechanism of the TCDD induced immune function alteration However in spite of the great number of investigations there is still considerable debate to the specific mechanism of these responses particularly in lymphoid tissue that may be used in biomarker studies in humans.

At present a programmed cell death (apoptosis, negative activation) is regarded not only as the basic mechanism of the negative selection of the thymocytes during their maturation and differentiation but also as one of the basic mechanisms of the immune response regulation and support of the immunological homeostasis. The apoptotic process is mediated via Fas-receptor (FasR)/Fas-ligand (FasL) interaction. The FasR/FasL-mediated apoptosis is involved in the activation-induced T-cell and B-cell death, T- and B- memory cells function, NK-cells and $CD8^+$ T-cytotoxic cells killing and maintenance of the immunological privilege In many cases the defectiveness of the coordination processes in the apoptosis system may result in the grave disoders of the immune system both towards the suppression (autoimmune pathology, immunooncology) and enhancement (immunodeficiencies) of the programmed cell death. The alteration of the apoptosis is now speculate as one of the mechanisms of the TCDD-induced immune function alteration.

In these studies we present the results demonstrating the influence of 2,3,7,8-TCDD on CYP4501A1activity, mitogenic response, apoptosis, cell viability and Fas receptor expression in the human peripheral blood lymphocytes cultures.

Material and Methods

The PBLs were obtained from 15 healthy volunteers – nonsmokers with unknown occupational exposure with the TCDD and related compounds. Mononuclear cells were isolated by fractionation on "Ficoll-Paque Plus" (Pharmacia Biotech) density gradient. The interphase cells were recovered and washed for two times (150 g, 10 min) with the RPMI-1640 (Sigma) and then resuspended in the culture medium - HEPES-modified RPMI-1640, containing 10% heat-inactivte FCS (Sigma), 50 μg/ml gentamicine (Sigma). The cells were adjusted to a concentration $1*10^6$ cells/ml. Assays were performed in mitogen stimulated lymphocytes and in lymphocytes stimulated with both mitogen and 10 nM 2,3,7,8–TCDD. The suspension was divided into two parts. Subsequently 10 ml of the cell suspension was distributed into each of two plastic Falcon T-30 flasks (25 cm^3) with loosened caps - 1) mitogen-stimulated culture (PHA-P (10 μg/ml, Difco) and PWM (5 μg/ml, Sigma) and 2) TCDD – treated culture in which TCDD was added simultaneously with mitogens. Cells were incubated for 72-hrs (37^0C, 5% CO_2).

EROD activity.

After the 72-hrs incubation lymphocytes were washed twice with warm RPMI-1640 and resuspended in 0.1 M EROD-assay potassium phosphate buffer (pH 7.60), contained $MgSO_4$ (5 mM) and BSA (2 mg/ml). The assay mixture contained 2.0 ml EROD-assay buffer, $2*10^6$ cells and 7-ethoxyresorufin (1.5 μM, Sigma). The reaction was initiated by the addition of NADPH (2.6 μmol)[7]. The assays were incubated at 37^0C for 45 min. The reaction was stopped by the addition of methanol (2 ml). Precipitated protein was removed (14000g 10 min) and the fluorescence of the supernatant was measured in a Bio-Rad VersaFluor fluometer (exitation wavelength 550 nm; emission wavelength 585 nm). The metanol-water solution of resorufin (Sigma) was used as a standard and the EROD activity was calculated as pmoles resorufin per 10^6 cell/min.

Blastogenesis assay.

[^{3}H] Thymidine (1 μCi per well) was added to the cultures 6 hrs befor the end of incubation. The cells were harvested on glass fiber filters and [^{3}H]Tdr incorporation (in cpm) was measured in a liquid scintilation counter (ISOCAP-300).

Cell viability and apoptotic cells determination.

Cells viability was measured by the 0.2% trypanblau staining. For the apoptotic cells determination cells were washed for two times with the warm 0.15 M PBS (pH 7.2) and resuspended in 0,15 M PBS-supravital staining solution containing 2.5 μg/ml Hoechst 33342 (Sigma). Cells were incubated for 15 min at 37°C, then washed single time with PBS and resuspended in the same medium. The suspension was analysed in the LUMAM IL6 LOMO microscope-fluometer (exitation wavelength 360 nm). The percentage of viable cells with the nuclear fragmentation (apoptotic cells) were count. For the early apoptosis determination cell were resuspended in the Annexin V-binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4) and then incubated with the human recombinante FITC-labled Annexin V (CALTAG Lab.). After the 10 min of incubation cells were washed, resuspended in binding buffer and the number of the Annexin V + cells were determined on the LUMAM IL6 LOMO microscope-fluometer.

Fas-receptor expression.

The FasR expression was determined by the indirect immunoflourescence method. Cells were washed for two times with 0.15 M PBS contained 3 mM sodium azide and 0.2% BSA

(Sigma) and resuspended in the same medium. Mice anti-human monoclonal antibodies (mAb) DX-2 clone (Caltag Lab.) against human FasR was used. Sheep F(ab)2 – FITC labled fragments against mouse IgG (ICN Pharmaceuticals) were used as "second" antibodies. LUMAM IL6 LOMO microscope-fluometer was used for evaluation of Fas-receptor bearing cells.

Results and Discussion.

The results are shown in table1 As it was determined by the tripan-blau staining the number of the dead cells in the TCDD-challenged cultures significantly increased. The mitogenic response slightly decreased. This results are agreed with our previous results[5] and those reported by Lozovatsky and coworkers[8] who obsereved the depression of mitogenic response of mononuclears in the TCDD and Benzo [a] anthracene treated-cultures. The loss of phospholipid membrane asymmetry, leading to exposure of Annexin V binding phosphatidylserin on the outside

Table 1. *The influence of 2,3,7,8-TCDD (10 nM) on the EROD-activity, cell viability, proliferation, apoptosis and Fas-receptor expression in the mitogen-stimulated human peripheral blood lymphocytes.*

	EROD-activity pmoles/10^6 cells/min)	Dead Cells,%	^{3}H-Tdr incorporation, cpm per 10^5 cells/min	Annexin V+ cells (early apoptosis), %	Cells with the nuclear fragmentation (late apoptosis) %	Fas-receptor expression, % of the CD95+ cells
Control (mitogen only)	0.09 (0.07-0.12)	5 (3.0-7.5)	57000 (38000-64000)	29 (20 – 40.8)	5.0 (3.1 – 6.55)	33 (24-43.3)
Mitogens + 10nM TCDD	0.20(0.14-0.33) P=0.0006	9 (6 – 12.5) P=0.005	48000 (25000-60000) n.s.	39.5 (15 – 47.5) n.s.	4.0 (3 – 6) P=0.08	46 (24 – 55) P=0.039

The data are expressed as Median (25 – 75%-ile). The Wilcoxon matched paired test was used for the statistic procedure.

of the plasma membrane is an early event in apoptosis[9]. The number of Annexin V+ cells in the mitogen+TCDD-treated cultures was higher than in the control cultures stimulated with the mitogens only (non significant) and the percentage of the Fas-receptor bearing cells during the coincubation with the TCDD increased (statistically significant). It is well known, that the Fas-receptor is the "professional" receptor for apoptogenic signals and its expression is one of the membrane markers of lymphocytes activation. However the percentage of cells with the nuclear fragmentation (late apoptosis) in the TCDD-treated cultures was the same as for control cultures. Moreover in some cultures the number of apoptotic cells was lower than in the mitogen-stimulated only. In the univariate analyses (Spearman's correlation) the statistically significant correlations between the basal EROD activity and TCDD-induced EROD activity and other parameters were not found. However in the multiple regression models an association between the cell viability (dependent variable) and basal EROD activity ($R = +0.44$, $P<0.10$) and induced EROD activity ($R = +0.60$, $P=0.02$) was found.

Recently it was shown that CYP4501A1 induction following in vivo and in vitro exposure to requires the activation of proteinkinase C[10]. On the other hand the PAHs-induced *suppression of mitogenesis was mediated through the increased phosphorylation of the Scr*-related protein tyrosine kinase (Lyn and Syk)[11]. Our data demonstate that TCDD – induced suppression of the mitogenic response did not correlate with EROD activity. These data are confirmed by Guijaeva and cowokers[12] in which different pathways for the activation signal transduction and for enzyme induction were shown. The assotiation between the non-apoptotic cell death and basal and TCDD-induced EROD activity proposed the possibility of the direct immunotoxic effect probably assotiated with the reactive oxygen generation during the CYP1A1 induction. The inhibition of apoptosis is now recognized as a key mechanism of action of the tumor-promoting agents 13). In rat hepatocytes TCDD is able to suppress apoptosis initiated by the UV light. This effect coincides with the suppression of the p53 response probably mediated by the AhR-dependent c-src activation and subsequent hyperphosphorylation of p53[14]. By the contrary in Daudy B-cells cultures terminal deoxynucleotidal transferase-mediated dUTP-biotin nick end labeling (TUNEL) analysis revealed a significant increase in the number of cells undergoing apoptosis and decreased bcl-2 expression in response to B[a]P[15]. In the present experiments the TCDD suppressed late apoptosis, but increased the Fas-receptor expression and the number of cells binding early apoptotic marker Annexin V. Thus the alteration of the apoptotic signal transduction may be supposed. Based on the resultes of our study it is tempting to speculate that there are different and possibly somewhat independent mechanisms of the CYP450 inducers - mediated abnormalities of the cell cycle regulation and apoptosis.

References.

1) Wright K. and Morgan E. (1991) Mol. Pharmacol. 39, 468 – 74.
2) Cribb A., Delaporte E., S. Kim., R. Novak. And K. Renton. (1993) J. Pharm. Exp. Ther. 268, 487 – 94.
3) Clark M., Bing B., Gottschall P. and J. Williams. (1995) Biochem. Pharmacol. 49, 97 – 104.
4) Holsapple M., Morris D., Wood S. and Snyder N. (1991) Annu. Rev. Pharmacol. Toxicol. 31, 73-100.
5) Narasimhan T., Craig A., Arellano L., Harper N., Howie L., Menache M., Birnbaum L. And Safe S. (1994) Fundam. Appl. Toxicol. 23, 598 – 607.
6) Sibiryak S., Amirova Z., Kurchatova N., Yusupova R. and Kruglov E. (1998) Organohal. Comp. 38, 267 – 70.
7) Thompson C., McCoy Z., Lambert J., Andries M. and G. Lucier (1989) Cancer. Res. 49, 6503 – 511.
8) Lozovatsky A., Ostashevsky V., Kozlov V., Tsyrlov I. And Perminova O. (1990) Bull. Exp. Biol. Med. (Russia) 109, 569 –71.
9) Koopman G., Reutelingspreger C., Kuijten G., Keehnen R., Pals S. and van Oers M. (1994) Blood, 84, 1415-20.
10) Stephen F., Drahushuk A. and Olson J. (1997) Toxicology, 124, 39 – 51.
11) Mounho B. and Burchiel S. **(1998)**Toxicol. Appl. Pharmacol., 149, 80-89.
12) Gujaeva E., Ostashkina N., Kondalenko V. and Koblyakov V. (1999) Biochemistry (Mosc.), 64, 929 – 32.
13) Wright S., Zhong J., Larrick J. **(1994)** FASEB J., 8, 654.
14) Schmitz H.-J., Wormer W. and Schrenk D. **1998,** Orhanogal. Comp., 37, 63 – 6.
15) Salas V., Burchiel S. **(1998)** Toxicol. Appl. Pharmacol,151, 367 – 76.