Original Article

Tumor necrosis factor-α inhibits effects of aryl hydrocarbon receptor ligands on cell death in human lymphocytes

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Abstract

Background: Activation of aryl hydrocarbon receptor (AhR) leads to diverse outcome in various kinds of cells. AhR activation may induce apoptosis or prevent of apoptosis and cell death. Recent studies suggest that apoptosis effects of AhR can be modulated by inflammatory cytokine like tumor necrosis factor alpha (TNF-α). In this study, we try to investigate the possible interaction of TNF-α with the 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), a ligand of AhR, on peripheral lymphocytes.

Materials and Methods: Human peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by discontinuous density gradient centrifugation on ficoll. Isolated PBMCs were divided into four groups: Control group, TNF-α administered group, TCDD administered group, co-administered group with TCDD and TNF-α. Cells were maintained for a week in lymphocyte culture condition. Then, TNF-α was added to group 2 and 4. Finally, apoptosis and necrosis were analyzed in all samples using flowcytometry.

Result: In group 4, the mean percent of necrosis and apoptosis in TCDD treatment groups was significantly larger than other groups; ($P < 0.05$). Furthermore, there was no significant difference between the mean percent of cell death in TNF-α administered group and TCDD administered group ($P > 0.05$). However, the mean percent of cell death in co-administered group with TCDD and TNF-α was significantly lower than other groups; ($P < 0.05$).

Conclusion: TNF-α could significantly inhibit effects of TCDD on lymphocytes apoptosis. Combination effects of TNF-α and TCDD on lymphocyte increase cell survival.

Key Words: Apoptosis, aryl hydrocarbon receptor, tetrachlorodibenzo-p-dioxin, tumor necrosis factor alpha

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Received: 06.08.2014, **Accepted:** 10.01.2015

Access this article online	
Quick Response Code:	Website: www.advbiores.net
	DOI: 10.4103/2277-9175.166163

INTRODUCTION

Aryl hydrocarbon receptor (AhR) has emerged as a major player in the immune system. AhR protein express by various cells of the immune system such as lymphocytes. Stimulation of AhR exerts potent immunosuppressive effect. Infectious diseases or cancer, in particular, are considered to be extremely

How to cite this article: Ghatrehsamani M, Soleimani M, Esfahani BA, Shirzad H, Hakemi MG, Mossahebimohammadi M, *et al*. Tumor necrosis factor-α inhibits effects of aryl hydrocarbon receptor ligands on cell death in human lymphocytes. Adv Biomed Res 2015;4:216.

affected by AhR activation.[1] For instance, AhR agonist are known to cause cancer, immunological deficiencies or death.[2] Immune cells also show extreme changes in cytokine secretion or function in response to AhR activation.[3] Due to its role in the immune system, AhR activation considered promising therapeutic target for immunological disease.[4]

The AhR is a cytosolic, basic helix-loop-helix ligand-activated transcription factor.[1,5] Polycyclic and halogenated aromatic hydrocarbon ligands activate ligand-dependent transcription factor AhR and result in multiple gene expression.[1,3] AhR has various kinds of ligand such as, 2, 3, 7, 8-tetra chlorodibenzop-dioxin (TCDD), xenobiotic and natural chemicals.[3] TCDD is one of the well-known ligand, which has shown to induce the functional activation of AhR.[6] When TCDD enters cells, binds AhR and lead to the conformational change. TCDD/AhR complex migrate to the nucleus, dimerize with AhR nuclear translocator (ARNT).[3,6] AhR/ARNT complex bind to xenobiotic response elements, which are located in the promoter regions of AhR response genes.[7] In response to agonist binding, AhR activate and lead to expression various kinds of genes such as CYP1A1 and CYP1B1 and several transferases.[8] Formerly, AhR linked to xenobiotic response but currently its role in the immune system attracted wider attention.[9] Because AhR has pivotal roles in gene expression and immune system, thereby it is important to understand the relationship of AhR activation and immune system.

Tumor necrosis factor (TNF-α) is a pro-inflammatory cytokine and has inflammatory and immunoregulatory effects.[10] TNF-α mediates its signaling due to tumor necrosis factor receptor 1 (TNFR-1) and tumor necrosis factor receptor 2 (TNFR-2). Both receptors mediate survival signal by ligation to TNF-α and activation of nuclear factor-κB (NF-κB), which cause anti apoptotic factor increment. NF-κB activation protecting cells from apoptosis. Although, TNFR-1, beside inflammatory response, mediates apoptotic signal.[11] In contrast to TNFR-2, TNFR-1 contains a death domain (DD). TNF-alpha/TNFR I ligation induces apoptotic signal by recruitment of adapter proteins containing homologous DD and lead to apoptosis.[12]

Many researchers have investigated the role of AhR in the immune system. TCDD has a major role in the suppression of several murine models of autoimmune diseases.[13] AhR activation also make individual more susceptible to infection.[14] It has found that AhR inhibit NF-κB signaling which decrease inflammatory cytokine production.[15,16] TCDD also, induces suppression of T cell-mediated immune responses.[17]

We hypothesize that inhibition of NF-κB signaling may shift TNF-α stimulated immune cell response to apoptosis rather than inflammation. Some studies support this hypothesis.[10,18] Our study goal was to elucidate that activation of AhR can induce apoptosis of T lymphocytes, inhibiting the NF-κB pathway and novel biological relationships that may underlie new potential drug in immunosuppressive treatment.

MATERIALS AND METHODS

Materials

Venous blood samples (n = 30) were collected in 50 ml Falcon tubes containing EDTA. The blood sample had each a volume between 10 and 15 ml and was transferred to cell culture laboratory in 4°C container. The study participants consisted of 30 healthy adult individuals with no history of clinical infection. The mean age of the volunteer donors was 27 years (range, 24–30 years). Ethical approval was granted by the Isfahan University of Medical Science Ethics Committee.

Reagents

Fetal bovine serum (FBS) and RPMI-1640 medium was purchased from Gibco, (New York, USA). Anti-CD3 monoclonal antibody (OKT3), Human IL-2 Recombinant Protein and Human TNF alpha Recombinant Protein were purchased from eBioscience (San Diego, CA, USA). annexin V-FITC/ propidium iodide (PI) assay kit was purchased from EXBIO Praha (Vestec, Czech Republic). Ficoll-Hypaque and TCDD was obtained from Supelco, Sigma-Aldrich Co. (Bellefonte, PA, USA).

Cell culture

The peripheral blood sample were prepared and cultured under sterile conditions similar to the method of Aggarwal *et al*.[19] Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-HyPaque, density gradient centrifugation (400 g for 20 min at 24°C). The PBMCs layer was washed three times with sterile phosphate-buffered saline (PBS). PBMCs were cultured in the presence of RPMI 1640 culture medium containing 2 mM L-glutamine, 20% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, purified anti-CD3 monoclonal anti-body (25 ng/ml) for 48 h which, followed by culture in 10 ng/ml of IL-2 containing medium for 4 days. These cells were also simultaneously treated with TCDD (10 nM/ml) or without TCDD. TCDD solutions were prepared by adding a constant amount of TCDD-containing FBS to the cell culture medium. The cells were cultured in a humidified 5% CO_2 incubator at 37°C. The final concentrations used in the cell treatments included 10 nM TCDD and 20% FBS. They were washed with

PBS and incubated further for 48 h in the presence or absence of TNF-α (10 ng/ml). After a week, the cells were divided into 4 groups by transferring the cell culture inserts to a Costar 12-well plate as shown in Table 1.

Flow cytometry

Cells were harvested, washed twice with PBS. Then cell's pellet resuspend in provided annexin V Binding Buffer and adjusted cells density to $2–5 \times 10^5$ cells/ml and followed by incubating annexin V-FITC and PI (15 min, RT) in the dark room. Then, cells were re-washed in binding buffer. Distribution of cells through apoptosis and necrosis were analyzed by flowcytometry using a Partek flowcytometer. Percentage of cells were calculated using flowjo software.

Statistical analysis

Regarding the normal distribution of data, One-way ANOVA was used to comparison means between four groups. Data were analyzed using Graphpad prism 5 for Windows (Version 5). A $P < 0.05\%$ was considered to be statistically significant.

RESULT

We analyzed the percent of lymphocyte apoptosis and necrosis by Annexin V/PI staining [Figure 1]. The impact of TNF-α on the lymphocyte necrosis induction by TCDD (at a maximum effective concentration of 10 nM) was investigated. It was observed that after 48 h of treatment, TNF-α partially inhibited induction of necrosis in combination with TNF-α (group 3) when compare to other groups ($P < 0.001$) [Figure 2a].

Table 1: Cell treatment

Groups	Cells and treatment
1	Only PBMC
2	PBMC treated by TNF-α
3	PBMC treated by TNF-α and TCDD
4	PBMC culture treated by TCDD

PBMC: Peripheral blood mononuclear cell, TNF-α: Tumor necrosis factor alpha, TCDD: 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin

As shown in Figure 2a, TCDD and TNF-α increase necrosis separately when compare to control, and combinatorial groups. Remarkably, the percentage of lymphocyte apoptosis from group 4 (treatment with TCDD) was significantly increased when compared with controls (1.963 ± 0.2 vs. 1.16 ± 0.005 $P < 0.01$), group 3, treatment occur with TCDD and TNF-α, (0.96 ± 0.2 vs. 1.16 ± 0.005 $P < 0.001$) and group 2, treatment with TNF-α, (1.27 ± 0.05 vs. 1.16 ± 0.005 $P < 0.001$) [Figure 2b]. We found no significant difference in the up regulation of apoptosis among group 2 and control. The percentage of lymphocyte apoptosis from group 3 was significantly decreased when compared with controls ($P < 0.01$), group 2 and group 4 ($P < 0.001$) [Figure 2b].

Tumor necrosis factor alpha potentiates survival induced by tetrachlorodibenzo-p-dioxin

We calculated cell death for each group by adding apoptosis percentage to necrosis percentage. Comparison between groups showed that treatment with TCDD and TNF-α significantly increase cell survival rather than other groups; $P < 0.001$ [Figure 3]. But, there was no significant difference between distinct TCDD treatment and TNF-α treatment in cell death $P > 0.05$. Also, the differences between each groups with control were significant ($P < 0.01$).

DISCUSSION

In the present study, we have shown that activation oh AhR with TCDD has antagonist effects on induction of apoptosis by TNF-α and increase cell survival.

Tetrachlorodibenzo-p-dioxin has the highest affinity for AhR, which is extremely conserved receptor from invertebrates to vertebrates. It has been shown, AhR has major roles in immunological behaviors.[20] AhR signaling influence immune cells differentiation like shifting T cells response to Treg and TH-17.[20,21] AhR signaling mediated Treg cells induction and can develop TH-17 cells.[22] AhR signaling also has an impact on cell proliferation, differentiation or

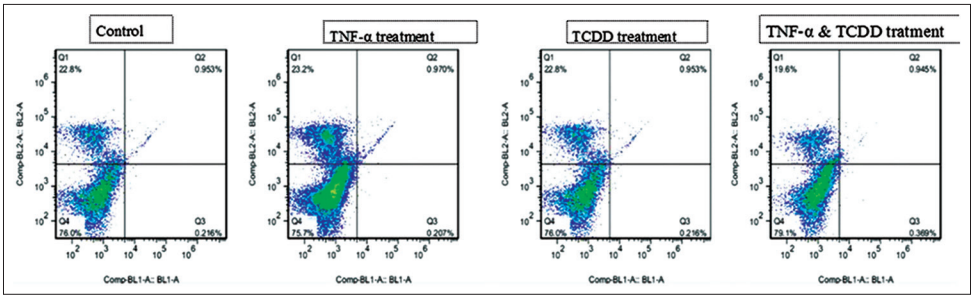

Figure 1: Flow cytometry dot plots of annexin V/propidium iodide in lymphocyte of four study groups Q1 show the percentage of apoptosis–Q2 show the percentage of apoptosis and necrosis–Q3 show the percentage of necrosis

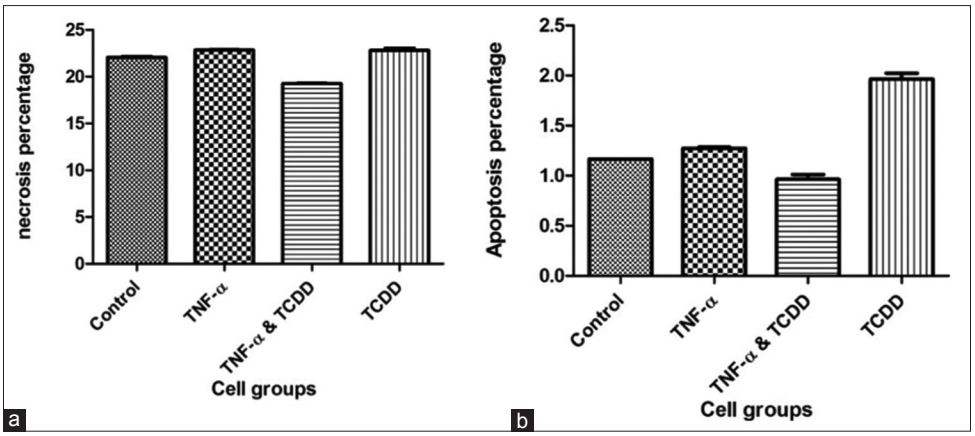

Figure 2: Comparison of the percentage of necrosis (a) and apoptosis (b) among the study groups

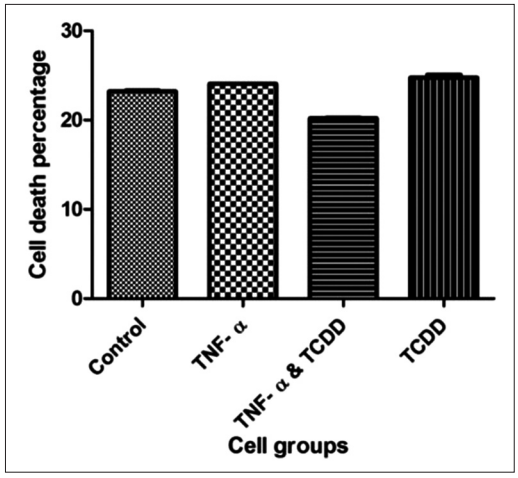

Figure 3: Comparison of the percentage of cell death among the study groups

apoptosis.[23] According to some studies, inflammation suppress cellular responses to AhR activation, whereas other studies have been shown that inflammatory mediators such as TNF-α increase expression of some AhR target genes.[24] However, currently there is not enough information on possible interactions between pro inflammatory cytokines such as TNF-α and AhR ligands in lymphocytes. Thus, we examine combined effects of AhR ligands and TNF-α on regulation of cell survival in peripheral blood lymphocytes.

We found that TNF-α and TCDD increase cell death in lymphocytes separately and we were able to demonstrate for the first time that AhR stimulation with TCDD in combination with TNF-α increase lymphocytes survival. The results of the study by Ito *et al*.[25] also showed that AhR induced apoptosis in Jurkat T cells by mechanism apart from fas expression. Base on the previous study, TNF-α induce lymphocyte apoptosis through activation of a family of cysteine proteases called caspases,[26,27] which is consistent with our findings.

Although some studies have shown the anti-apoptotic role of AhR in Mouse hepatoma cells, human osteosarcoma, mouse embryonic fibroblasts, and primary lung fibroblasts,[28] which is inconsistent with our findings. Maybe AhR has a different effect on various kinds of cells.

The characterization of apoptosis and necrosis was analyzed by flowcytometery. Our results revealed the decrement level of apoptosis and necrosis in the lymphocyte group treated with TCDD and TNF-α significantly ($P > 0.001$). These results may suggest that AhR decrease apoptosis in association with TNF-α. In the agreement with our present data, Umannova *et al*.[24] demonstrated that TNF-α significantly increased proliferation and survival in rat liver stem like cells after incubation with TCDD. This would suggest that there is a kind of mutual functional between inflammatory cytokine such as TNF-α and AhR. It seems AhR and TNF-α, together shift response to cell survival and suppress apoptotic effects of their own. AhR may shift TNFR signaling to activation of NF/kB and that explain the lack of apoptotic effects of TNF-α. Some experiments found that proinflammatory cytokines like TNF-α suppress the expression of CYP1A1, which increase by AhR activation.[29,30] CYP activation might involve regulation of cell cycle progression and apoptosis.[8] This might indicate the mechanism that TNF-α inhibit AhR in the way that result in apoptosis induction.

Altogether, our results suggested that activation of AhR in inflammatory condition may have various kinds of effects on lymphocytes rather than normal conditions.

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Source of Support: Isfahan University of Medical Science,
Conflict of Interest: None declared.