Original Article

2,3,7,8-tetrachlorodibenzo-p-dioxin decrease expression of aryl hydrocarbon receptor in peripheral lymphocyte of β-thalassemia major patients

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Abstract

Background: β-thalassemia major is a hereditary disease with inefficient erythropoiesis. Level of inflammatory cytokine is elevated in these patients. In this study, we investigate the effect of aryl hydrocarbon receptor (AhR) ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), on the expression of inflammatory mediators in β-thalassemia major patient's lymphocytes.

Materials and Methods: Peripheral blood mononuclear cells of patients and healthy participants was isolated and cultured in favor of lymphocytes increment. Based on the treatment, we divided the cell into four groups. The orders of group's treatments were no treatment, tumor necrosis factor-α (TNF-α) treatment, TNF-α and TCDD treatment, TCDD treatment in Group 1–4, respectively. After cell culture, we extracted the cells RNA and converted them to cDNA. Real-time polymerase chain reaction was performed to assessment relative expression of caspase-1, NLRP3, and AhR. We compared all patient groups with equal healthy (control) groups.

Results: Results showed that expression of caspase-1 in patients (Groups 1 and 2) was significantly lower than healthy individuals ($P < 0.05$). Although, no significant difference was found (Groups 1, 2, and control) in AhR gene expression ($P > 0.05$). Expression of AhR in other groups of patients (3 and 4) was significantly lower than control groups ($P < 0.05$). Expression of caspase-1 in Group 4 was significantly larger than the control group ($P < 0.001$).

Conclusions: We show here that chronic inflammation decrease caspase-1 expression and exposure of human lymphocytes to TCDD promote caspase-1 expression. Furthermore, activation of AhR with TCDD decreases AhR expression in lymphocytes of β-thalassemia major disease.

Key Words: 2,3,7,8-tetrachlorodibenzo-p-dioxin, β-thalassemia major, aryl hydrocarbon receptor, caspase-1

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INTRODUCTION

β-thalassemia major is an inherited anemias resulting from absent or low-level synthesis of β-globin chains of hemoglobin A in erythropoietic cells.[1] The ineffective erythropoiesis and hemolysis leads to the conspicuous anemia seen in the β-thalassemia major disease, which can be corrected with repeated blood

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transfusion.[2] Major side-effect of iron transfusion is iron overload,[3] which can lead to immune system dysfunction and increase infections.[4,5] After heart failure, infection is a major cause of death in patients with β-thalassemia major.[6] One of the mechanism that iron overload effects on the immune system is an increment of reactive oxygen species, which result in the production of pro-inflammatory cytokines through activation of nuclear factor-κB (NF-κB).[2] Thus, the levels of inflammatory cytokines like tumor necrosis factor-α (TNF-α) are elevated in β-thalassemia major patients.[7,8] TNF-α is a pro-inflammatory cytokine that can initiate NF-κB activation signal. NF-κB has a major role in activation of the inflammasome.[9] It is becoming increasingly obvious that in the lack of infection, TNF-α supply the essential signals to prepare the NLRP3 inflammasome complex for activation.[10]

In order to assessment the potential impact of environmental factors on inflammasome in β-thalassemia major patients, we have focused on the ligand-dependent transcription factor aryl hydrocarbon receptor (AhR), which responds to environmental factors and plays a major role in the immunological responses.[11] The AhR is a member of the basic helix-loop-helix nuclear translocator, which modulate immune responses.[12] Previous study has shown suppression of AhR leads to severe inflammation together with an elevation of interleukin-1β (IL-1β), a main cytokine of inflammation.[12] Activation of AhR inhibits IL-1β secretion that is controlled by the NLRP3 inflammasome and caspase-1.[12,13] A recent paper has shown that AhR expression in immune cells depends on NF-κB activation.[14]

In this study, we try to compare the effect of AhR activation on expression of NLRP3 inflammasome components, caspase-1, and AhR in lymphocytes of β-thalassemia major patients versus healthy individual's lymphocytes. We predicted that investigation into the effects of NF-κB on the expression of inflammatory mediators is vital towards a better understanding of *β*-thalassemia major pathogenesis. Thus, our study goal was to clarify novel immunological relationships that may reveal new therapeutic ways in chronic inflammation conditions.

MATERIALS AND METHODS

Subjects

Venous blood samples of β-thalassemia major patients (n = 30) that hospitalized at Seyedo Shohada Medical Center of Isfahan were collected in 50 ml Falcon tubes (sorfa) containing EDTA. The volume of blood samples was between 10 and 15 ml. The control groups (normal groups) of study participants consisted of 30 healthy adult individuals with no history of specific and chronic clinical infection. The mean age of all 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 (USA). Anti-CD3 monoclonal antibody (OKT3), Recombinant human IL-2 protein, and recombinant human TNF-α protein were purchased from eBioscience (San Diego, CA, USA). Ficoll-hypaque and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and the agarose was obtained from Supelco, Sigma-Aldrich Co., (Bellefonte, PA, USA). Trizol reagent, one-step real-time polymerase chain reaction (RT-PCR) kits, and DNA ladder was purchased from Invitrogen (Carlsbad, CA). RNeasy Mini kit, QuantiTect Reverse Transcription Kit, and SYBR Green PCR master mix was purchased from QIAGEN Co., (GmbH, Hilden, Germany).

Cell culture

The peripheral blood samples of the β-thalassemia major patients and healthy individuals (control participant) were prepared and cultured under sterile conditions, which shift cell to lymphocytes. After separation, the peripheral blood mononuclear cells (PBMCs) with ficoll, cells were prepared to culture 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 antibody (25 ng/ml), and 10 ng/ml of IL-2 containing medium for 6 days.[15] These cells were also simultaneously treated with TCDD (10 nM/ml) or without TCDD. The cells were cultured in a humidified 5% CO_2 incubator at 37°C (10 ng/ml). On day 7, the cells were washed with phosphate-buffered saline and incubated further for 48 h in the presence or absence of TNF-α. In fact, they were divided into four groups for patients and equally four groups for healthy by transferring the cell culture inserts to a Costar 12-well plate.

Group 1: No treatment - Group 2: TNF-α treatment - Group 3: TNF-α and TCDD - Group 4: TCDD treatment.

Extraction of total RNA and cDNA synthesis

After cells culture, isolated PBMCs were homogenized in Trizol. RNA was extracted from the homogenized lysates using Qiagen RNeasy mini kit following the kit instructions. To check for successful genomic DNA removal, each sample was tested in RT-PCR for β-actin with and without reverse transcriptase using one-step RT-PCR kit. Quality of the RNA was checked by visualization of the 28S: 18S ribosomal RNA ratio on a 1% agarose gel. Once samples were confirmed as DNA

free, Total RNA was used and first-strand cDNA was synthesized using QuantiTect Reverse Transcription Kit in accordance with the manufacturers' instructions.

Real-time polymerase chain reaction

All the primers were exon junction and were designed by Beacon software (version 8, Stratagene). The sequences of the PCR primer pairs are as follows: AHR forward primer, ATTGAAGAAGCCACTGGTC; AHR reverse primer, CAGCAGACACCTTAGACGAC; NLRP3 forward primer, GCTTCAGGTGTTGGAATTAGAC; NLRP3 reverse primer, TCAGCACTTCACAGAACATCAT; caspase-1 forward primer, CACTGCTTCGGACATGAC; caspase-1 reverse primer, ACATGAACACCAGGAACG; GAPDH forward primer, CTCTCTGCTCCTCCTGTTCG; GAPDH reverse primer, ACGACCAAATCCGTTGACTC. cDNA was amplified using a qRT-PCR SYBR Green master mix kit. RT-PCR was carried out on a Rotor-Gene 6000 (Corbett). GAPDH was used to normalize total cDNA. Efficiency for each test was designed to be 1. The optimized assay consisted of a 13 μL reaction containing 6.5 μL SYBR green master mix, 10 pM of primers (1 μL), 1 μL of cDNA template and 4.5 μl H_2O. PCR cycling used the following conditions: 95°C for 2 min and 40 cycles of 95°C for 15 s, 58°C for 20 s and 72°C for 30 s. The ending step of PCR is performed to obtain the dissociation curve. All cDNA samples were analyzed in triplicate and negative controls without template were added each time. The PCR products were taken to run agarose gel for validating the specificity of the final products. A 100 base pair DNA ladder was used as the size determiner. Differences in the data for the mRNA expression of genes were analyzed using REST software 2.0.13 (Qiagen).

RESULTS

We evaluated and compared the expression of caspase-1, NLRP3, and AhR in the peripheral lymphocytes of β-thalassemia major patients and healthy individuals. All targeted genes normalized with GAPDH gene. Cells differentiation state was checked by flow cytometry looking at the common lymphocytes marker. Expression of caspase-1 was decreased in patient Group 1 and Group 2 rather than similar healthy groups ($P < 0.05$) Figure 1. AhR expression showed no significant change between these groups [Figure 1]. TCDD treatment decreased AhR expression in Group 3 and Group 4 ($P < 0.05$) [Figure 2]. No significant difference was observed in the expression of caspase-1 between co treatments of TNF-α and TCDD groups (Group 3) [Figure 2]. However, the rate of caspase-1 induction appeared to be significantly increased in TCDD treatment group (Group 4) compared to control group ($P < 0.001$) [Figure 2]. NLRP3 expression in all groups was undetectable by RT-PCR assessment.

DISCUSSION

Our study demonstrated that TCDD affect the expression of AhR in β-thalassemia major patient's PBMCs. TCDD treatment can slightly increase caspase-1 production, but significantly inhibit AhR expression.

Aryl hydrocarbon receptor has major roles in the immune system. It mediates the proliferation, differentiation, cytokines secretion of immune cells, and lead to shifts the Th1/Th2 balance toward Th1 dominance.[16] The functions of AhR in Treg-cells (T-cells) are based on the specific ligand bound to the receptor. For instance, binding of TCDD to AhR suppresses the immune system in autoimmune diseases by promoting the development of regulatory T-cells.[17] Although in inflammatory conditions, expression of AhR increases in immune cells, AhR reduces inflammation by interaction with signaling molecules like STAT1. AhR also inhibits the transcriptional activity of NF-κB.[18] however, NF-κB is

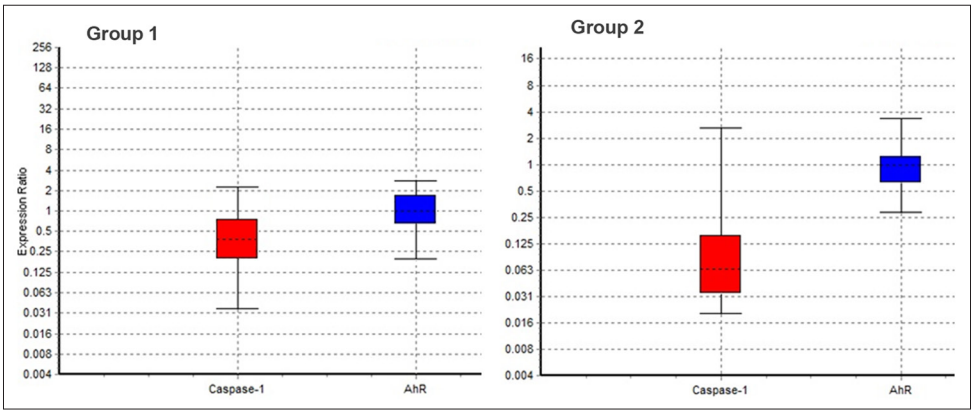

Figure 1: Relative expression of normalized caspase-1 and aryl hydrocarbon receptor (AhR) in β-thalassemia major patient's lymphocytes in comparison to control groups. The relative expression of normalized target genes. In Group 1 and 2, expression of caspase-1 decrease in comparison to equal control groups with 95% confident intervals. No significance difference was seen in expression of AhR among groups

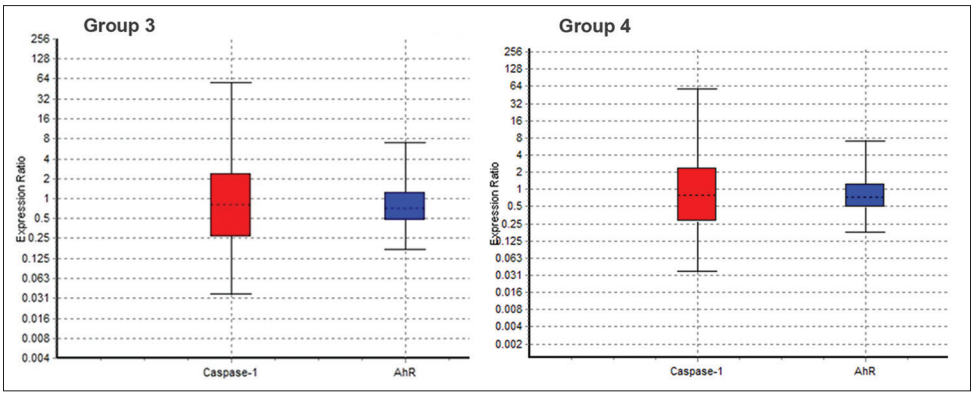

Figure 2: Relative expression of normalized caspase-1 and aryl hydrocarbon receptor (AhR) in Group 3 and 4 rather than healthy participants. In contrast to control groups, relative expression of normalized AhR decreased in both groups and expression of caspase-1 increased in Group 4 (only 2,3,7,8-tetrachlorodibenzo-p-dioxin treatment) significantly $P < 0.001$. No significant difference was seen in caspase-1 expression between patients and control. The confidence interval is 95%

an essential component inducing the expression of AhR and the regulation of AhR-dependent genes expression in immune cells.[14]

However, currently there is not enough information on possible interactions of AhR on lymphocytes in chronic inflammatory conditions. Thus, we examined combine effects of AhR ligands and TNF-α on the expression of AhR and mediators of the inflammasome in peripheral blood lymphocytes.

Here, we demonstrated that caspase-1 expression decreased in peripheral lymphocytes of β-thalassemia major patients and treatment of lymphocytes with TCDD increased expression of this inflammasome mediator in lymphocytes. These results imply that AhR plays an important role in the production of IL-1β and the host resistance against bacterial infections.

Previous work has shown that AhR deficiency enhanced susceptibility to bacterial infection. They did not investigate the caspase-1 expression, one of the major mediator for IL-1β production in infection diseases.[17] On the other hand, several study[11,19] have shown that activation of AhR suppress production of pro-inflammatory cytokines and has a major role in autoimmune diseases. However, they did not compare the levels of expression of caspase-1 in lymphocytes, which may have caused the discrepancy between our and their results. Vogel *et al*.[20] showed that activation of AhR may lead to increment pro-inflammatory cytokines which is consistent with our findings. These results suggested that, activation of AhR may elevate host resistance to bacterial infections in chronic inflammation conditions like β-thalassemia major. Expression of NLRP3 was not detectable in all groups. It seems NLRP3 did not express in peripheral human lymphocytes or expression this component of inflammasome complex depends on the specific stimulants.

One of the striking findings of this study is that expression of AhR decreased after lymphocytes treatment with TCDD. To our knowledge, it has not been previously recognized that AhR expression decreased after ligand stimulation. One explanation for that is inflammation induces expression of AhR and TCDD inhibit inflammatory responses that lead to AhR expression. In agreement with our present data Champion *et al*.[21] demonstrated that NF-κB has a critical role in AhR expression. Furthermore, AhR lead to suppression of NF-κB expression.[22]

The present study showed positive correlations between AhR activation and caspase-1 expression. This result suggested that TCDD and inflammation cytokines would be relevant factors in regulating the transcriptional levels of AHR and the elevated AHR may be involved in the expression of inflammation mediators.

REFERENCES

1. Ribeil JA, Arlet JB, Dussiot M, Moura IC, Courtois G, Hermine O. Ineffective erythropoiesis in ß -thalassemia. ScientificWorldJournal 2013;2013:394295.
2. Mahmoud SM, Sahar B. Aziz Evaluation of certain inflammatory markers in transfusion dependent β-thalassemic patients. Tikrit J Pharm Sci 2013;9:6-7
3. Rund D, Rachmilewitz E. Beta-thalassemia. N Engl J Med 2005;353:1135-46.
4. Cunningham-Rundles S, Giardina PJ, Grady RW, Califano C, McKenzie P, De Sousa M. Effect of transfusional iron overload on immune response. J Infect Dis 2000;182 Suppl 1:S115-21.
5. Pieracci FM, Barie PS. Iron and the risk of infection. Surg Infect (Larchmt) 2005;6 Suppl 1:S41-6.
6. Wang SC, Lin KH, Chern JP, Lu MY, Jou ST, Lin DT, *et al.* Severe bacterial infection in transfusion-dependent patients with thalassemia major. Clin Infect Dis 2003;37:984-8.

7. Kuppusamy UR, Tan JA. Chelation therapy with desferrioxamine does not normalize ferritin level but attenuates oxidative damage and improves total antioxidant level in Malaysian Chinese beta-thalassaemia major patients. West Indian Med J 2011;60:3-8.
8. Morabito N, Russo GT, Gaudio A, Lasco A, Catalano A, Morini E, *et al.* The "lively" cytokines network in beta-thalassemia major-related osteoporosis. Bone 2007;40:1588-94.
9. Álvarez S, Muñoz-Fernández MÁ. TNF-A may mediate inflammasome activation in the absence of bacterial infection in more than one way. PLoS One 2013;8:e71477.
10. Chow MT, Duret H, Andrews DM, Faveeuw C, Möller A, Smyth MJ, *et al.* Type I NKT-cell-mediated TNF-a is a positive regulator of NLRP3 inflammasome priming. Eur J Immunol 2014;44:2111-20.
11. Di Meglio P, Duarte JH, Ahlfors H, Owens ND, Li Y, Villanova F, *et al.* Activation of the aryl hydrocarbon receptor dampens the severity of inflammatory skin conditions. Immunity 2014;40:989-1001.
12. Ikuta T, Kobayashi Y, Kitazawa M, Shiizaki K, Itano N, Noda T, *et al.* ASC-associated inflammation promotes cecal tumorigenesis in aryl hydrocarbon receptor-deficient mice. Carcinogenesis 2013;34:1620-7.
13. Beamer CA, Seaver BP, Shepherd DM. Aryl hydrocarbon receptor (AhR) regulates silica-induced inflammation but not fibrosis. Toxicol Sci 2012;126:554-68.
14. Vogel CF, Khan EM, Leung PS, Gershwin ME, Chang WL, Wu D, *et al.* Cross-talk between aryl hydrocarbon receptor and the inflammatory response: A role for nuclear factor-kB. J Biol Chem 2014;289:1866-75.
15. Gupta S. A road to ruins: An insight into immunosenescence. Basic Biol Clin Impact Immunosenescence 2003;13:173.
16. Hanieh H. Toward understanding the role of aryl hydrocarbon receptor in the immune system: Current progress and future trends. Biomed Res Int 2014;2014:520763.
17. Nguyen NT, Hanieh H, Nakahama T, Kishimoto T. The roles of aryl hydrocarbon receptor in immune responses. Int Immunol 2013;25:335-43.
18. Kimura A, Abe H, Tsuruta S, Chiba S, Fujii-Kuriyama Y, Sekiya T, *et al.* Aryl hydrocarbon receptor protects against bacterial infection by promoting macrophage survival and reactive oxygen species production. Int Immunol 2014;26:209-20.
19. Zhu C, Xie Q, Zhao B. The role of AhR in autoimmune regulation and its potential as a therapeutic target against CD4 T cell mediated inflammatory disorder. Int J Mol Sci 2014;15:10116-35.
20. Vogel CF, Sciullo E, Matsumura F. Involvement of RelB in aryl hydrocarbon receptor-mediated induction of chemokines. Biochem Biophys Res Commun 2007;363:722-6.
21. Champion S, Sauzet C, Bremond P, Benbrahim K, Abraldes J, Seree E, *et al.* Activation of the NF kB pathway enhances AhR expression in intestinal caco-2 cells. ISRN Toxicol 2013;2013:792452.
22. Tian Y, Ke S, Denison MS, Rabson AB, Gallo MA. Ah receptor and NF-kappaB interactions, a potential mechanism for dioxin toxicity. J Biol Chem 1999;274:510-5.

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