Aromatic hydrocarbon receptor provides a link between smoking and rheumatoid arthritis in peripheral blood mononuclear cells

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Abstract

Objective

Epidemiology shows that smoking plays a central role in rheumatoid arthritis (RA). The aim of this study was to evaluate the potential relationship between smoking, aromatic hydrocarbon receptor (AHR) and RA susceptibility.

Methods

We performed a hospital-based, case-control study of patients with RA and healthy controls. Expressions of AHR, cytochrome P4501A1(CYP1A1), aromatic hydrocarbon receptor repressor (AHRR) genes were assessed in peripheral blood mononuclear cells (PBMCs) and cultured cells using real-time PCR. The response of PBMCs to the AHR agonist, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and cigarette smoke extract (CSE) were cultured in vitro.

Results

AHR and its downstream gene expressions were demonstrated in smoking rheumatoid PBMCs and non-smoking patients with significantly higher expression in smoking patients. The observation was consistent with the sensitivity of RA PBMCs to TCDD and CSE stimulation demonstrated in vitro.

Conclusion

Our study shows that smoking may be involved in the pathogenesis of RA by the AHR pathway.

Key words

smoking, rheumatoid arthritis, aromatic hydrocarbon receptor

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Introduction

Rheumatoid arthritis (RA) is a complex chronic antoimmune disease mainly with polyarthritis that affects approximately 1% of the population (1) and the causes of which mainly due to environmental and genetic factors. Evidence showed that different environmental factors, such as cigaratte smoking act as trigger stimuli for the development of RA, leading to synovial hyperplasia and bone destruction, and patients with RA who continue to smoking will have a worse disabilities and higher disease activity (2). Tobacco smoking is considered as a primary aetiologic factor in RA. Cigarette smoke-derived chemical substances such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), benzo[a]pyrene (BaP), and 3-methylcholanthrene (3-MC) are agonists with a high affinity (3).

Aromatic hydrocarbon receptor (AHR) is a ligand-dependent transcription factor which combines xenobiotic response elements (XRE) in the existence of its ligands to regulate gene expression and affect the development and differentiation of cells, and the deficiency of AHR or its hyperfunction will both result in antoimmune disorders (4, 5). Cyto-chrome P4501A1 (CYPlAl) is a most studied responsive gene that belongs to a member of cytochrome P450 (CYP) family of enzymes, and the AHR repressor (AHRR) is a characteristic factor responsive to AHR activiation. There is evidence that AHRR and CYP1A1 gene expressions are significantly high expressed when AHR is activated by its ligands such as TCDD (3, 6, 7, 8). In this study, we analyse the effect of tobacco smoking on expressions of AHR, AHRR and CYP1A1 in RA peripheral blood mononuclear cells (PBMCs) to give a preliminary presumption that the reasons of smoking in the development and progression of RA and further to provide several clinical therapy.

Materials and methods

Study subjects

Thirty-one smoking patients and thirty-one non-smoking patients were diagnosed with RA from Anhui Provincial Hospital, Anhui Medical University between May 2014 and June 2016 according to the American Rheumatism Association 1987 revised criteria for the classification. During the time, the blood samples of fifteen healthy smoking donors and fifteen non-smoking controls were obtained from healthy examination centre of the hospital. The included criteria for RA group: (a) definite diagnosis; (b) never having leflunomide therapy for all patients; (c) actively smoking when the blood sample was taken – including those who were daily; (d) C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) in the normal range, Disease Activity Score 28 (DAS28) less than 3.2 and having no joint swelling and RA pain. Individuals with other diseases were excluded from the study. Patient smoking status at the time of peripheral blood collection was established. Informed consent was obtained from all subjects.

Preparation of CSE and TCDD

Cigarette smoke extract (CSE) was prepared based on the method of Vassallo *et al*. (9). Smoke was pulled into a 10-ml plastic syringe with a three-way stopcock through a tube containing 10ml RPMI 1640 pre-warmed (eight pulls per cigarette). A common Chinese brand of cigarette was used in this study. TCDD was purchased from Maipu biological company of Shanghai. The experimental (CSE-treated and TCDD-stimulated) groups *in vitro* were used for each dosage 1mg /ml, 10 mg / ml and 0.1μmol/ml, 1μmol/ml.

Cell isolation and cell culture

10-ml peripheral blood from RA patients and healthy controls was collected in heparin-anticoagulant tubes, and 6 ml of the nonsmoking patients and healthy controls were cultured. Ficoll-Hypaque technology was applied to isolate PBMCs of all blood samples. All cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum,100U/ml of penicillin G, 100 mg/ml of streptomycin and were stimulated with CSE and TCDD mimicked the effect of smoke for 72 hours at 37°C in air containing 5% CO2. And 72 hours later, cells were collected to prepare for the RT-PCR.

RNA extraction and preparation of cDNA

Total RNA was severely extracted using a Trizolreagent (Invitrogen California, USA) according to the guide of manufacture. The specific primers were designed and synthesised by Takara Biotechnology (Tokyo, Japan). The primers used were as follows: for AHR, forward 5'-ATACCGAAGACCGAGCTGAAT-3'and reverse5'-CCAGCAGACACC-TTAGACGAC-3'; for AHRR, forward 5'-AGGTTTGGTTGGCAGG-ACT-3' and reverse 5'-GCTCAGATGGTTG-GCTGTTC-3'; for CYP1A1, forward 5'-CTCTGTGAG-GGTGCT-GACTG-3' and reverse 5'-TATC-CTGGGCTGTCTCTTCC-3';for β-actin, forward 5'-TGGCACCCA-GCACAATGAA-3' and reverse 5'-CTAAGTCATAGTCCGCCTAGA-AGCA-3'; The PrimeScript RT reagent Kit (Takara Biotechnology, Japan) was used to perform the synthesis of cDNA according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR)

Assays were performed using ABI 7500 Fast Real-Time PCR System (Applied Biosystems, California, USA), and the PCR was completed in a 20-ul reaction system containing 1.6ul primers (0.8ul-Forward primer and Reverse primer, respectively), 2ul cDNA, 0.4ul ROX Referencedye II, 6ul dH2O, and 10ul SYBR Premix Ex TaqTM II. $2^{-\Delta\Delta ct}$ was used to compute the expression value of AHR, AHRR and CYP1A1.

Laboratory measurements

For all the RA patients, ESR, CRP and anti-cyclic citrullinated peptide (anti-CCP) antibodies were measured.

Statistical analysis

Statistical analysis was carried out by the Statistical Package for Social Sciences 10.0 software e (SPSSInc.; 2000). The differences Of AHR, AHRR and CYP1A1mRNA expression levels between subjects or groups were analysed by Mann-Whitney U-test. Sperman correlation analysis was used in our study. A *p*-value less than 0.05 was considered to be statistically significant.

Table I. Age and gender of patients with RA and controls.

Group		Male (n)	Female (n)	Age (Y) Mean ± SD	Course	*p* value
RA	Smoker	14	17	(50.9±1.0)	0.5-30y	*p*>0.05
	Non-Smoker	15	16	(47.5±7.7)	0.25-38y	
Healthy humans	Smoker	8	7	(48.8±0.9)	-	*p*>0.05
	Non-smoker	8	7	(47.2±1.3)	-	

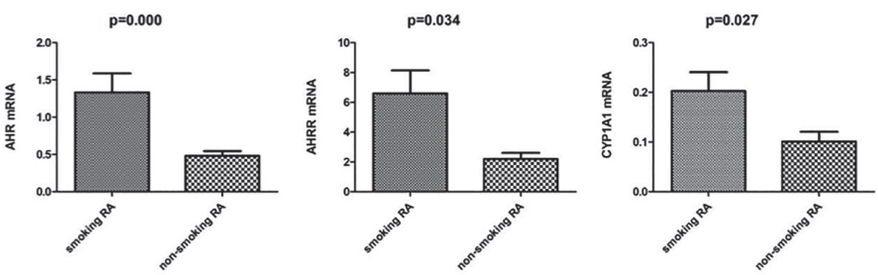

Fig. 1. The mRNA of PBMCs in RA patients between smoking group and non-smoking group.

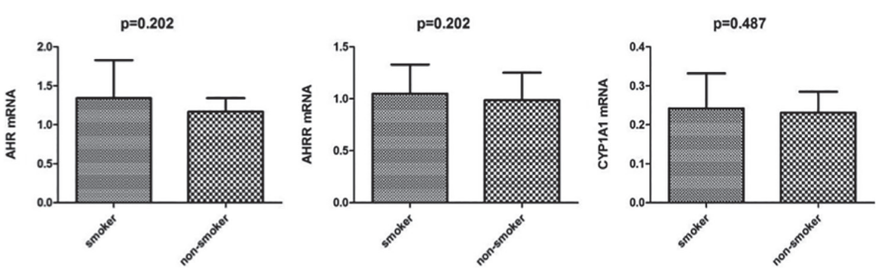

Fig. 2. The mRNA of PBMCs in healthy humans between smoking group and non-smoking group.

Results

Characteristics of the study population

As shown in Table I, age and gender are matched between cases and controls. The frequency of groups was appropriate on age and sex (*p*=0.622 and 0.264, respectively).

The expression levels of AHR, AHRR and CYP1A1 mRNA in PBMCs from experimental groups and controls

AHR expression was present in PBMCs. Expression of AHR responsive gene, CYP1A1 and AHRR were used to establish the activation status in PBMCs.AHR, AHRR and CYP1A1 mRNA expression levels in PBMCs were assessed by RT-PCR.As shown in Fig. 1, AHR, AHRR and CYP1A1 expression levels were significantly increased in smoking patients with RA than that in non-smoking patients [0.88 (0.48,1.41) *vs*. 0.38 (0.20,0.73), *p*=0.000; 2.87 (1.27,11.0) *vs*. 1.40 (0.80,3.18), *p*=0.034; 0.11(0.057,0.34) *vs*. 0.078 (0.027,0.14), *p*=0.027]. However, no significant differences were found between smoking healthy subjects and non-smoking healthy humans [0.24 (0.15,1.92) *vs*. 1.01 (0.67, 1.91), *p*=0.202; 0.83 (0.22,1.55) *vs*. 0.71 (0.33,1.04), *p*=0.899; 0.10 (0.01,0.42) *vs*. 0.21 (0.09,0.28) *p*=0.487] (Fig. 2).

The mRNA expressions of AHR, AHRR and CYP1A1 mRNA in PBMCs from subjects in vitro with differential TCDD and CSE sensitivity

Levels of AHR, AHRR, CYP1A1 were higher from PBMCs of RA with 1mg/ml(C1), 10mg /ml(C2) CSE and 1μmol/ml (T2) TCDD stimulation when compared to controls. However, no significant difference about the expressions of AHR, CYP1A1, AHRR were found from RA with 0.1μmol/ml (T1) TCDD-treated when compared with controls. Moreover, we failed to detect such significant differences between smoking healthy person and non-smoking group *in vitro*. The details are shown in Figs. 3-4.

Relationships between AHR, AHRR, CYP1A1 mRNA expressions and clinical parameters in the smoking patients with RA

We conducted Spearman's correlation to assess the association between AHR, AHRR, CYP1A1 mRNA expressions and smoking status in patients. Unfortunately, no significant correlation was found between them (r=0.028, p=0.891; r=0.058, p=0.871; r=-0.151, p=0.462) (Fig. 5).

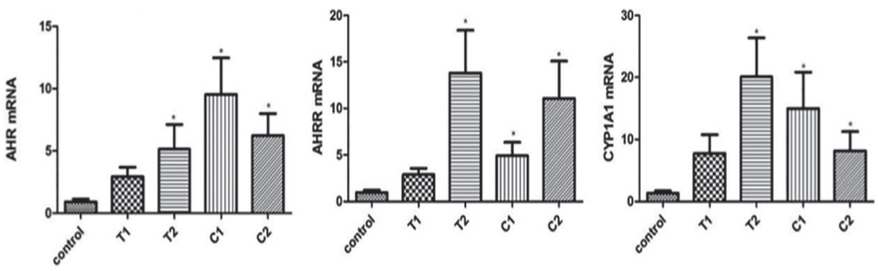

Fig. 3. The mRNA of PBMCs in RA patients between groups *in vitro* with different doses of CSE and TCDD. The figure shows significant difference between groups with dosage of 1mg/m l(C1), 10mg /ml(C2) CSE and 1μmol/ml (T2) TCDD (*p<0.05),and no significant difference with dosage of 0.1μmol/ml TCDD.

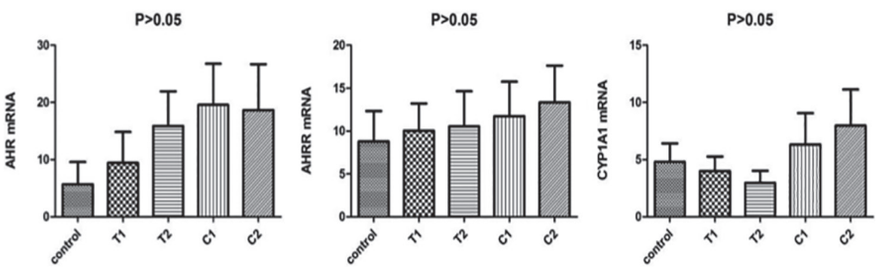

Fig. 4. The mRNA of PBMCs in healthy humans between groups *in vitro* with different doses of CSE and TCDD.

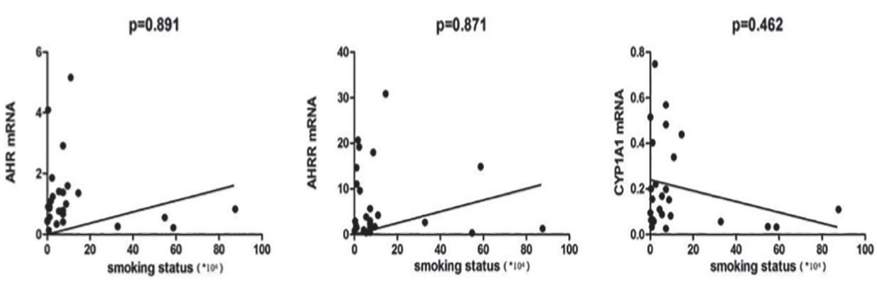

Fig. 5. Relationships between AHR, AHRR, CYP1A1 mRNA expressions and smoking status in the smoking patients with RA. The horizontal axis is represented the total number of cigarettes.

Discussion

RA is the most common type of chronic autoimmune arthritis. However, the pathogenesis of RA primarily was unknown, and the causes of which mainly due to the interactions of environmental and genetic factors. Environmental factors such as TCDD, BAP and 3-MC are high potent agonists that may combine with AHR to activate the signalling pathways and further regulate T cells, macrophages, dendritic cells (DCs), B cells as well as pro- and anti-inflammatory cytokines; eventually lead to antoimmune diseases including RA (10).

Two main pathways are involved with the activation of AHR (11, 12, 13). One classic way is that AHR is existed in the cytoplasm with its chaperonins and maintains inactive. When the compounds in tabacoo TCDD included combine with AHR, the chaperone proteins are released and the [AHR-agonist] migrate to nucleus binding to the transcription factor AHR nuclear translocator protein (Arnt).And at last, [AHR-agonist-Arnt] binds to the specific DNA sequences called xenobiotic-response elements (XRE) to regulate the expression of its downstream gene such as CYP1A1, UGT1A1, AHRR (11). On the other hand, AHR interacts with other signalling ways to mediate immune responses. Meanwhile, AHR could activate transcription factor NF-κB through making an inducible expression of regulatory T cells or suppressing the development of TH17 cells to regulate autoimmune diseases (12, 13). AHR probably activate its downstream gene to further produce or inhibit an inflammation in the pathogenesis of disease with different agonists. Data presented here will provide novel hypothesis into the relationship between AHR and RA.

Currently, a large number of studies have studied the pathogenesis of RA. Epidemic data points to smoking is a strong environmental risk factor in the development of RA and leads to worse disease outcomes (14, 15). Further more, paper reported therapeutic effects might be influenced by smoking status (16). Previous study (17) showed anti-inflammatory drug leflunomide is an agonist of AHR, therefore all the donors were never having leflunomide to avoid affecting outcomes. Tamaki *et al*. (18) demonstrated that AHR agonists, such as 3-MC, BaP, and TCDD, up-regulate the levels of AHR, AHRR and CYP1A1 in a human-like synoviocyte cells. Brauze *et al*. (19) reported AHR, AHRR and CYP1A1 mRNA were highly expressed in hepal when mice treated with TCDD compared to the control group. Kazantseva (3) showed AHR and CYP1A1 mRNA were increased in synovium of smoking RA patients compared with controls. Adachi *et al*. (8) also showed AHR activated gene CYP1A1 mRNA was increased in synoviocyte of RA *in vitro* with cigarette smoke condensate. Given these findings, that indicate cigarette smoking containing AHR ligands positively correlates with RA. To date, this is the first study to analyse the associations among smoking, AHR expression and RA in PBMCs. Understanding the mechanism about smoking and AHR in RA might allow us to treat RA.

The results presented in this paper AHR,

AHRR, CYP1A1 mRNA were highly expressed in PBMCs of smoking patients than controls, at the same time, the mRNAs were also significantly increased of RA patients *in vitro* with dosage of 1mg/ml, 10mg /ml CSE and 1μmol/ml TCDD treated, what were consistent with a list of researches (3, 20). But Such findings at *vivo* and *vitro* of PBMCs may indicate that the activation of AHR by smoking and thereby contribute to the severity of RA. However, the expressions were not obviously increased with 0.1μmol/ml TCDD, which may include insufficient stimulated time, the dosage of TCDD and so on. Also, we failed to detect any correlation between AHR, AHRR, CYP1A1 expressions and smoking status in PBMCs of RA patients. The reasons for this outcome are probably multifactorial. One possible explanation is some polymorphisms in AHR, AHRR and CYP1A1 genotype may influence the expression of them under our consideration. Others may include the fat, infection, using drugs and so on. The hypothesis needs further follow-up cohort studies.

In conclusion, our data suggests that cigarette smoke-induced changes in PBMCs responses have the potential to be relevant to RA. That is the risk of RA from smoking likely relates to a mechanism of AHR activation. It may broaden our horizons in the aetiology of RA and provide new insights into characterisation of RA. However, larger studies and more studies may be needed to elucidate these results in the future study. Furthermore, the mechanisms of these effects may need functional studies to clarify this issue.

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