Journal of Dermatological Science 72 (2013) 9–15

Contents lists available at SciVerse ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Reduction of CC-chemokine ligand 5 by aryl hydrocarbon receptor ligands

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ARTICLE INFO

Article history:
Received 20 January 2013
Received in revised form 9 April 2013
Accepted 29 April 2013

Keywords:
Aryl hydrocarbon receptor
Dioxins
Chemokines
CC-chemokine ligand 5
Keratinocytes

SUMMARY

Background: The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that recognizes a large number of xenobiotics, such as polycyclic aromatic hydrocarbons (PAHs), dioxins, and some endogenous ligands. Despite numerous investigations targeting AhR ligands, the precise physiological role of AhR remains unknown.
Objective: We explored novel AhR target genes, especially focused on inflammatory chemokine.
Methods: We treated (1) HaCaT, a human keratinocyte cell line, (2) normal human epidermal keratinocytes (NHEKs), and (3) mouse primary keratinocytes with AhR ligands, such as 6-formylindolo[3,2-b]carbazole (FICZ; endogenous ligand) and benzo[a]pyrene (BaP; exogenous ligand). Then, we detected mRNA and protein of chemokine using quantitative RT-PCR and ELISA. We next clarified the relationship between AhR and chemokine expression using AhR siRNA. In addition, we measured serum chemokine levels in patients with Yusho disease (oil disease), who were accidentally exposed to dioxins in the past.
Results: We identified CC-chemokine ligand 5 (CCL5), a key mediator in the development of inflammatory responses, as the AhR target gene. AhR ligands (FICZ and BaP) significantly reduced CCL5 mRNA and protein expression in HaCaT cells. These effects were observed in NHEKs and mouse primary keratinocytes. AhR knockdown with siRNA restored CCL5 inhibition by AhR ligands. In addition, AhR ligands exhibited a dose-dependent suppression of CCL5 production induced by Th1-derived cytokines. Finally, serum levels of CCL5 in patients with Yusho disease, were significantly lower than in controls.
Conclusion: Our findings indicate that CCL5 is a target gene for AhR, and might be associated with the pathology of dioxin exposure.

1. Introduction

Dioxins and polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (BaP) are environmental contaminants to which humans and wildlife are widely exposed. These pollutants tend to accumulate in higher animals and may interfere with the immune system [1,2]. Dioxin and PAH toxicity have been linked, at least in part, to activation of the aryl hydrocarbon receptor (AhR), a ligand-activated basic helix–loop–helix transcription factor. Upon ligand binding, cytoplasmic AhR translocates to the nucleus and dimerizes with the AhR nuclear translocator (ARNT). The ligand-activated AhR/ARNT complex then binds specific promoter elements called xenobiotic response elements, altering the expression of target genes presumed to contribute to dioxin and PAH toxicity [2]. Several reports have shown that dioxins and PAHs induce multiple inflammatory genes including cytokines and chemokines such as IL-1α, TNF-α, IL-8, and CC-chemokine ligand 1 (CCL1) and 2 in an AhR-dependent manner in vivo and in vitro [3–7]. Numerous other exogenous compounds, such as plant

Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; BaP, benzo[a]pyrene; CCL, CC-chemokine ligand; FICZ, 6-formylindolo[3,2-b]carbazole; NHEK, normal human epidermal keratinocyte; PAHs, polycyclic aromatic hydrocarbons; PCB, polychlorinated biphenyls; TCDD, 2,3,4,8-tetrachlorodibenzo-p-dioxin.

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0923-1811/$36.00
http://dx.doi.org/10.1016/j.jdermsci.2013.04.031

polyphenols (e.g., resveratrol and curcumin) and synthetic drugs (e.g., ketoconazole) activate AhR, while some are thought to be AhR antagonists [8,9]. Although the physiological role of AhR remains largely unclear, endogenous AhR ligands have been proposed, such as 6-formylindolo[3,2-b]carbazole (FICZ) and bilirubin [10,11]. We previously reported that BaP induces IL-8 production from normal human epidermal keratinocytes (NHEKs) via the AhR signaling pathway, and that ketoconazole inhibits BaP-induced IL-8 production from NHEKs [7,9]. In this study, we further screened the production of various inflammatory chemokines in response to AhR ligands (FICZ and BaP) from human and mouse keratinocytes. We also determined serum chemokine levels in patients with Yusho disease (oil disease), who were accidentally exposed to dioxins in the past.

2. Materials and methods

2.1. Reagents and antibodies

FICZ was obtained from Enzo Life Sciences (Plymouth Meeting, PA); BaP and dimethyl sulfoxide (DMSO) were obtained from Sigma–Aldrich (St Louis, MO). Anti-AhR rabbit polyclonal antibody (H-211) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin rabbit polyclonal antibody (#4967) was purchased from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were provided by Jackson ImmunoResearch Laboratories (West Grove, PA).

2.2. Cell culture

HaCaT cells, representing a human keratinocyte cell line, were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics. HaCaT cells were plated on 6-well plates, and at sub-confluence were treated with AhR ligands (FICZ (0.1, 1, 10, or 100 nM) or BaP (0.01, 0.1, 1, or 10 μM)) or vehicle (DMSO). NHEKs, obtained from Clonetics-BioWhittaker (San Diego, CA), were maintained in serum-free keratinocyte growth medium (Lonza, Walkersville, MD) supplemented with bovine pituitary extract, recombinant epidermal growth factor, insulin, hydrocortisone, transferrin, and epinephrine (Lonza, Walkersville, MD). NHEKs were plated on 24-well plates, and were treated with AhR ligands (FICZ (0.1, 1, 10, or 100 nM) or BaP (0.01, 0.1, 1, or 10 μM)) or vehicle (DMSO) at sub-confluence.

2.3. Preparation of mouse epidermal cell suspension

RPMI-1640 supplemented with 10% heat-inactivated FBS, antibiotics, and 50 μM 2-mercaptoethanol (Sigma–Aldrich) were used as complete medium. Ear skin specimens from female Balb/c mice (7–8 weeks old) were treated with 0.5% trypsin (Sigma–Aldrich) in phosphate-buffered saline (PBS; 30 min at 37 °C); this separated the epidermis from the dermal connective tissue. Epidermal sheets were agitated gently in complete medium with 0.05% deoxyribonuclease I (Sigma–Aldrich); the epidermal cell suspension was obtained following filtration through a cell strainer with a 40-μm pore size (BD Falcon, San Jose, CA). Mouse primary keratinocytes were plated onto 6-well plates, and at sub-confluence were treated with AhR ligands (10 nM FICZ or 1 μM BaP) or vehicle (DMSO). The protocol was approved by the Committee of Ethics on Animal Experiments in the Graduate School of Medical Sciences, Kyushu University.

2.4. Combined stimulation with TNF-α and IFN-γ

Cells were plated on 6-well plates, and at sub-confluence were exposed to TNF-α (10 ng/mL) and IFN-γ (10 ng/mL) in the presence or absence of AhR ligands (FICZ or BaP) for indicated times. After incubation, the medium was extracted for ELISA.

2.5. Real-time quantitative RT-PCR

Total RNA was isolated from HaCaT cells using the RNeasy Mini kit (Qiagen, Valencia, CA). Quantitative real-time RT-PCR was performed with PrimeScript RT reagent and SYBR Premix Ex Taq II (Takara Bio, Ohtsu, Japan) according to the manufacturer's instructions. PCR amplifications were performed with the following cycling conditions: 95 °C for 30 s, for 40 cycles at 95 °C for 5 s (denaturation step), at 60 °C for 20 s (annealing/extension steps). The cycle threshold (Ct) for each amplification was normalized to β-actin (internal control). Normalized gene expression was expressed as the relative quantity of gene-specific mRNA compared with control mRNA (fold induction). Oligonucleotide primers are listed in Table 1.

2.6. Immunoblotting

Protein lysates from HaCaT cells were isolated with lysis buffer (25 mM HEPES, 10 mM $Na_4P_2O_7{\cdot}10H_2O$, 100 mM NaF, 5 mM EDTA, 2 mM Na_3VO_4, 1% Triton X-100) and analyzed by SDS-PAGE on a 10% polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and probed with specific antibodies. Immunological bands were identified with HRP-conjugated secondary antibodies followed by visualization with SuperSignal west pico chemiluminescence substrate (Pierce, Rockford, IL).

2.7. ELISA

Cell-culture supernatants were cleared by centrifugation and analyzed for the presence of immunoreactive CCL5 protein by using the Quantikine human or mouse CCL5/RANTES ELISA kit (R&D System, Minneapolis, MN) as directed by the manufacturer. Serum chemokine levels (CCL2, CCL5, CXCL9, and CXCL10) were also measured in 232 patients diagnosed with Yusho disease living in Fukuoka, Japan and 96 age- and

Table 1
Primer sequences used for real-time quantitative RT-PCR.

Gene (human)	Primer sequence (5′–3′)	
	Sense	Antisense
CYP1A1	TAGACACTGATCTGGCTGCAG	GGGAAGGCTCCATCAGCATC
CCL2	CCCCAGTCACCTGCTGTTAT	TGGAATCCTGAACCCACTTC
CCL5	TCTGCGCTCCTGCATCTG	GGGCAATGTAGGCAAAGCA
CXCL9	TTCCTCTTGGGCATCATCTTGCTGG	AGTCCCTTGGTTGGTGCTGATGCAG
CXCL10	CAAACTGCGATTCTGATTTGCTGCC	TGCTGATGCAGGTACAGCGTACGGT
β-Actin	ATTGCCGACAGGATGCAGA	GAGTACTTGCGCTCAGGAGGA

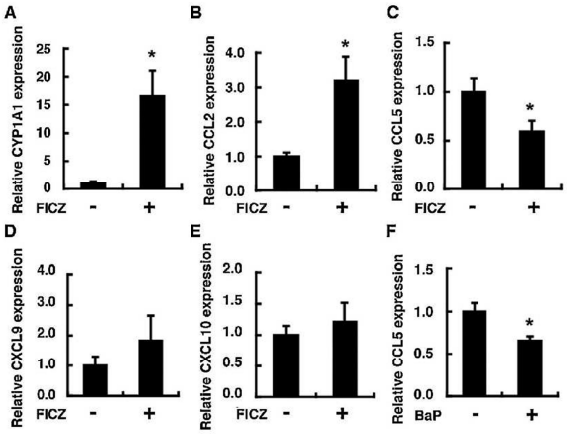

Fig. 1. AhR ligands reduced expression of CCL5. (A–E) HaCaT cells were treated with 10 nM FICZ for 3 h and total RNA was extracted. CYP1A1 (A), CCL2 (B), CCL5 (C), CXCL9 (D), and CXCL10 (E) mRNA expression was measured by real-time quantitative RT-PCR. mRNA levels were normalized to β-actin (internal control). (F) HaCaT cells were treated with 1 μM BaP for 3 h and total was RNA extracted. CCL5 mRNA expression was measured by real-time quantitative RT-PCR and normalized to β-actin. Data are presented as mean ± S.E. ($n = 4$ per group). $^{*}P < 0.05$ by unpaired t test.

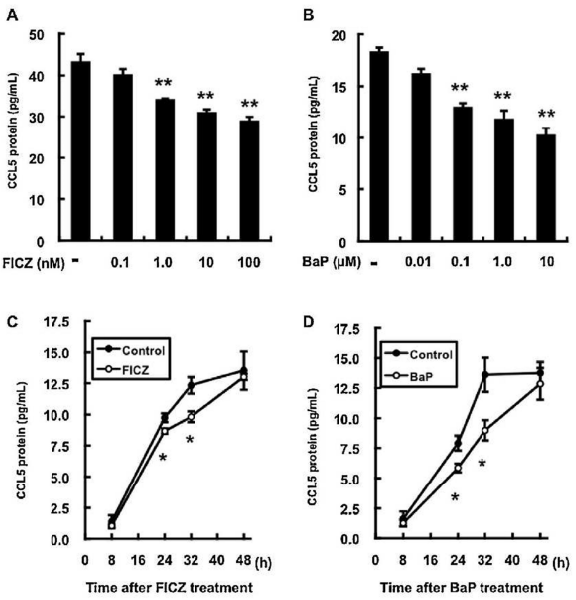

Fig. 2. Reduction of CCL5 protein levels in response to AhR ligands. (A and B) HaCaT cells were treated with FICZ (A) or BaP (B) for 24 h, and culture supernatants were collected. CCL5 protein was measured by ELISA. Data are presented as mean ± S.E. ($n = 4$ per group). $^{**}P < 0.01$ vs. control (DMSO only), assessed by one-way ANOVA (C and D) HaCaT cells were treated with 10 nM FICZ (C) or 1 μM BaP (D) for indicated time, and culture supernatants were collected. CCL5 protein was measured by ELISA. Data are presented as mean − S.E. ($n = 4$ per group). $^{*}P < 0.05$ by unpaired t test.

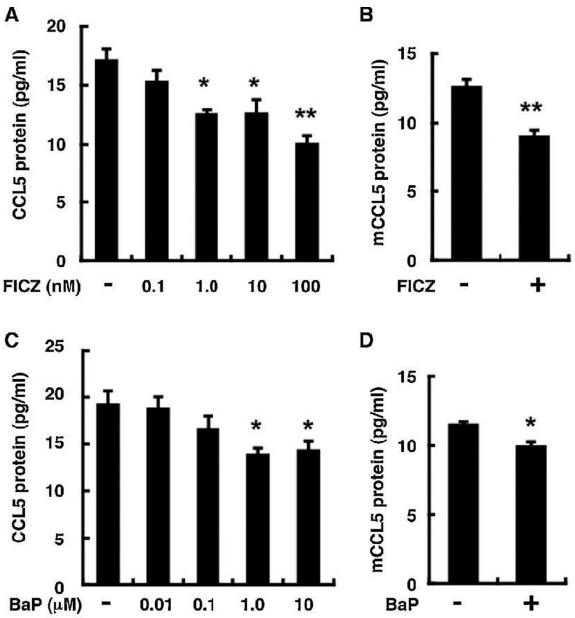

Fig. 3. Reduction of CCL5 protein levels in response to AhR ligands in NHEK and mouse primary keratinocytes. NHEKs were treated with FICZ (A) or BaP (C) for 24 h and culture supernatants were collected. CCL5 protein was measured by ELISA. Mouse primary keratinocytes were treated with 10 nM FICZ (B) or 1 μM BaP (D) for 24 h and culture supernatants collected. CCL5 protein was measured by ELISA. Data are presented as mean ± S.E. ($n = 4$ per group). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control (DMSO only), assessed by one-way ANOVA (A and C) or unpaired t test (B and D).

residency-matched normal individuals. Samples were acquired from 2005 to 2009 and stored at −80 °C to await analysis. Absorbance was measured using an iMark microplate absorbance reader (Bio-Rad, Hercules, CA), and the concentration of chemokines was determined in each sample by comparison to a standard curve. The study protocol was approved by the institutional ethics committee of Kyushu University Hospital and signed informed consent was obtained from each subject prior to study enrolment. Blood concentrations of dioxins in each patient were measured at the Fukuoka Institute of Health and Environmental Sciences (Fukuoka, Japan) as described elsewhere [12].

2.8. AhR siRNA transfection

AhR siRNA (s1200) and control siRNA (Negative Control #1) were purchased from Ambion (Austin, TX) and transfected as required into HaCaT cells using the HiPerFect Transfection kit (Qiagen) in accordance with the manufacturer's instructions.

2.9. Statistical analysis

Data are presented as mean ± S.E. Significance of the differences between groups was assessed using the Student's unpaired two-tailed t test (when 2 groups were analyzed), one-way ANOVA (for ≥3 groups), or the Mann–Whitney U-test (for human samples). A P value of <0.05 was considered statistically significant.

3. Results

3.1. AhR ligands reduce CCL5 expression in HaCaT cells

We verified the transcriptional activation of CYP1A1 (Fig. 1A) and CCL2 (Fig. 1B) by FICZ in HaCaT cells, a human keratinocyte cell line, as positive controls. We then assessed whether FICZ influenced the expression of other inflammation-related chemokines, such as CCL5 (CCR5 ligand) and CXCL9 and 10 (CXCR3 ligands). FICZ significantly reduced the expression of CCL5, but not that of CXCL9 and CXCL10 (Fig. 1C–E). BaP, another AhR ligand, also significantly reduced CCL5 mRNA expression (Fig. 1F). Thus, AhR ligands likely influence the transcription of CCL5.

3.2. AhR ligands inhibit CCL5 protein expression in HaCaT cells

We next examined of CCL5 protein levels in HaCaT cells treated with FICZ. Dose-dependent reduction of CCL5 protein was observed in HaCaT cells treated with FICZ (Fig. 2A) and BaP (Fig. 2B). To elucidate the time course of CCL5 suppression by AhR ligands, HaCaT cells were cultured in FICZ or BaP for up to 48 h. FICZ (Fig. 2C) or BaP (Fig. 2D) significantly decreased CCL5 protein in a time-dependent manner and restored it after 48 h. Our results show that AhR ligands FICZ or BaP, reduce not only mRNA but also protein levels of CCL5 in HaCaT cells.

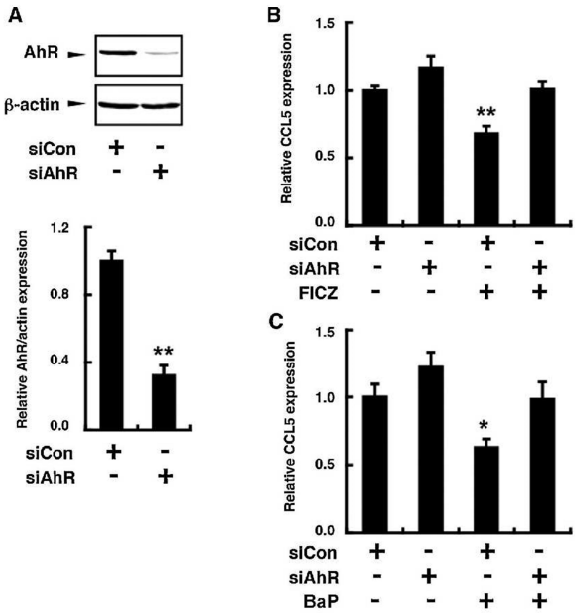

Fig. 4. AhR-dependent reduction of CCL5 expression in response to AhR ligands in HaCaT cells. (A) HaCaT cells were transiently transfected with AhR siRNA or control siRNA for 48 h and whole cell lysates were extracted. The relative amount of AhR was normalized to β-actin. Values were quantified using Image Gauge software. Data are presented as mean ± S.E. (n = 3 per group). $^{**}P < 0.01$ assessed by unpaired t test. (B and C) HaCaT cells were transiently transfected with AhR siRNA for 48 h, then treated with 10 nM FICZ (B) or 1 μM BaP (C) for 3 h. Total RNA was extracted and CCL5 mRNA expression measured by real-time quantitative RT-PCR. mRNA levels were normalized to β-actin. Data are presented as mean + S.E. (n = 4 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control (control siRNA + DMSO), assessed by one-way ANOVA.

3.3. AhR ligands inhibit expression of CCL5 in NHEKs and mouse primary keratinocytes

To determine whether AhR ligands reduce CCL5 expression in other types of keratinocytes, we used NHEKs and mouse primary keratinocytes. FICZ significantly reduced CCL5 protein in a dose-dependent manner in NHEKs (Fig. 3A) and mouse primary keratinocytes (Fig. 3B). BaP also reduced CCL5 protein levels in NHEKs and mouse primary keratinocytes (Fig. 3C and D). Thus, AhR ligands suppressed CCL5 expression in keratinocytes.

3.4. AhR ligands reduce CCL5 expression via AhR

To investigate the involvement of AhR in FICZ or BaP-mediated inhibition of CCL5 expression, we employed AhR siRNA silencing. Reduction of AhR protein levels was confirmed in AhR

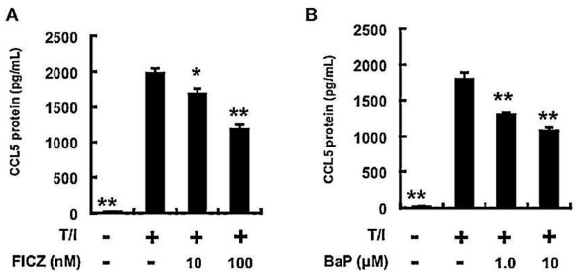

Fig. 5. AhR ligands inhibited CCL5 induced by the combined stimulation of TNF-α and INF-γ. HaCaT cells were stimulated with TNF-α and INF-γ (T/I) in the presence or absence of FICZ (A) or BaP (B) for 24 h, and culture supernatants were collected. CCL5 protein was measured by ELISA. Data are presented as mean ± S.E. (n = 4 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. T/I only, assessed by one-way ANOVA.

Table 2
Serum levels of chemokines in dioxin-exposed and control samples.

	CCL5 (ng/mL)	CCL2 (pg/mL)	CXCL9 (pg/mL)	CXCL10 (pg/mL)
Yusho (n = 232)	40.8 ± 1.5	388.2 ± 28.7	152.2 ± 8.7	125.0 ± 6.0
Control (n = 96)	59.3 ± 3.2	321.1 ± 10.7	159.5 ± 14.1	121.5 ± 12.6
P	<0.0001	0.14	0.66	0.78

Data are presented as mean ± S.E.
P values were assessed by Mann–Whitney analysis.

siRNA-treated cells compared with control siRNA-treated cells (Fig. 4A). Intriguingly, AhR knockdown with siRNA abolished the suppression of CCL5 expression by either FICZ or BaP (Fig. 4B and C). These results suggest FICZ or BaP might reduce CCL5 expression via AhR.

3.5. AhR ligands inhibit TNF-α/IFN-γ-induced CCL5 expression

Keratinocytes can be induced to produce CCL5 protein when incubated with a combination of Th1-derived cytokines (TNF-α and IFN-γ). Therefore, we examined whether AhR ligands decrease the production of CCL5 in HaCaT cells that have been stimulated with TNF-α and IFN-γ. Consistent with previous report, stimulation with TNF-α and IFN-γ increased CCL5 protein in HaCaT cells (Fig. 5A and B). Interestingly, TNF-α/IFN-γ-induced CCL5 expression was inhibited by FICZ in a dose-dependent manner (Fig. 5A). BaP also suppressed CCL5 expression induced by TNF-α/IFN-γ (Fig. 5B).

3.6. Serum levels of CCL5 are reduced in dioxin-exposed human samples

Mean ages of the Yusho patients and controls were 70.8 ± 12.0 years and 66.1 ± 11.3. Blood levels of dioxins in Yusho patients, in particular 2,3,4,7,8-pentachlorodibenzofuran, were significantly higher than in controls (170.5 ± 16.5 pg/g lipid vs. 15.7 ± 0.9 pg/g lipid). Serum levels of CCL5 in Yusho patients were significantly lower than in controls (Table 2). Although not significant, serum levels of CCL2 in Yusho patients tended to be higher than in the controls. There was no difference in the serum concentrations of other chemokines between Yusho patients and controls.

4. Discussion

AhR ligands directly or indirectly modulate chemokine expression. For example, intraperitoneal administration of 2,3,4,8-tetrachlorodibenzo-p-dioxin (TCDD) in C57/BL6 mice induced expression of CXCL1 and CCL2 mRNAs in the liver, thymus, kidney, adipose tissue, and heart [13]. Furthermore, in vitro studies have shown that AhR ligands, such as TCDD, polychlorinated biphenyl 126 (PCB126), PCB77, or BaP, upregulate the expression of CCL1, CCL2, CXCL13, and IL-8 in various cell lines and primary cell cultures [3–7]. In contrast, CCL5 expression appears to be inhibited by AhR ligands. In utero exposure to TCDD in male rats reduced CCL5 expression in the testes [14]. Furthermore, gene array analysis revealed that CCL5 expression was inhibited in $CD4^+$ T cells isolated from TCDD-treated mice [15]. In this study, we also showed significantly reduced production of CCL5 protein in human and mouse keratinocytes stimulated with FICZ and BaP. These AhR ligands markedly suppressed CCL5 expression induced by Th1-derived cytokines (TNF-α and IFN-γ). In addition, as previously shown in our preliminary study [16], we demonstrated that serum levels of CCL5 in dioxin-exposed patients were significantly lower than in normal subjects. Yusho disease (oil disease) is a serious form of food poisoning caused by consumption of rice oil contaminated with dioxins and related organochlorines in western Japan in 1968, involving at least 1900 individuals [17]. Patients with Yusho disease have suffered from various symptoms such as general malaise, arthralgia, chloracne, and peripheral neuropathy. Blood levels of dioxins in these patients remain high [18].

CCL5 plays a pivotal role in maintenance of the inflammatory response through its ability to attract T lymphocytes, monocytes, natural killer cells, and basophils, which leads to temporo-spatial expansion of the inflammatory infiltrate [19]. CCL5 expression is involved in a variety of diseases including arthritis, SLE, diabetes, and glomerulonephritis [20–23]. We showed that AhR ligands suppressed Th1-derived cytokine-induced CCL5 production, suggesting that AhR ligands would inhibit to some extent the infiltration of inflammatory cells. On the other hand, low baseline CCL5 was reported to be an independent predictor of cardiac mortality in a cohort of male patients undergoing coronary angiography [24].

Numerous basic and epidemiological studies have shown that dioxins and related organochlorines may increase the risk for cardiovascular diseases. For example, chronic exposure to dioxins (TCDD or PCB126) led to a dose-dependent increase in the incidence of degenerative cardiovascular lesions in rats [25]. The International Agency for Research on Cancer Cohort, consisting of 36 cohorts from 13 countries, followed 21,863 dioxin-exposed workers for more than 20 years. A significant association between dioxin exposure and ischemic heart disease was detected in these workers (relative risk, 1.67; 95% CI, 1.23–2.26) [26]. Although further studies are needed to clarify the precise clinical significance of our data, the observed reduction of serum CCL5 in dioxin-exposed patients may be related to the increased incidence of cardiovascular diseases in such patients.

Acknowledgments

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology and from the Ministry of Health, Labour and Welfare, Japan.

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