International Immunopharmacology 36 (2016) 111–117

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Benzopyrene, a major polyaromatic hydrocarbon in smoke fume, mobilizes Langerhans cells and polarizes Th2/17 responses in epicutaneous protein sensitization through the aryl hydrocarbon receptor

Chien-Hui Hong [a,b,1], Chih-Hung Lee [c,d,1], Hsin-Su Yu [e,f], Shau-Ku Huang [f,*]

[a] Department of Dermatology, National Yang Ming University, Taipei, Taiwan
[b] Department of Dermatology, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan
[c] Department of Dermatology, Kaohsiung, Chang Gung Memorial Hospital, Kaohsiung, Taiwan
[d] Department of Dermatology, Chang Gung University, Taoyuan, Taiwan
[e] Department of Dermatology, Kaohsiung Medical University, Kaohsiung, Taiwan
[f] National Health Research Institute, Miao-Li, Taiwan

ARTICLE INFO

Article history:
Received 30 December 2015
Received in revised form 6 April 2016
Accepted 7 April 2016
Available online xxxx

Keywords:
Benzopyrene
Epicutaneous protein sensitization
Langerhans cells
Aryl hydrocarbon receptor

ABSTRACT

Background: Atopic dermatitis (AD) is a common disease with genetic and environmental interactions. We previously reported lifetime exposure to cigarette smoke is associated with adult-onset AD. Aryl hydrocarbon receptor (AhR) is important in regulating environmental exposure to xenobiotics, including benzopyrenes (BP), a major polycyclic aromatic hydrocarbon (PAH) present in cigarette smoke. However, how AhR regulates immune responses in sensitization phase of AD remained elusive.
Methods: We investigated how BP affects epicutaneous sensitization response through AhR axis. We compared AhR expression in skin from AD patients and healthy controls. We measured immune responses (Langerhans cell migration and T cell polarization in epicutaneous Ova sensitization in mice with or without AhR defect.
Results: We found AhR and ARNT (AhR nuclear translocator) are upregulated in AD skin. BP exposure increases Langerhans cell migration, and increases IL-5, IL-13, and IL-17 levels when lymph node cells were re-challenged with Ova. The increased cytokine levels were attenuated in AhR defected mice. AhR agonists (BP and ITE) decreased *E*-cadherin expression, while AhR antagonist (CH223191) increased it in human primary keratinocytes.
Conclusions: These results suggested AhR interacts with BP to polarize T cell responses, along with Langerhans cell migration. This study revealed a regulatory mechanism how cigarette smoking affects atopic sensitization through the benzopyrene-AhR interaction.

1. Introduction

Atopic dermatitis (AD) is a common chronic relapsing inflammatory skin disease which usually heralds other types of atopic diseases [1], including bronchial asthma, allergic rhinitis, and allergic conjunctivitis. AD affects about 10% of the general population [2]. Studying early events of the "atopic march" contributes to the development of targeting therapy for atopic diseases [3]. The molecular pathogenesis of AD may involve abnormal epidermal differentiation and aberrant immune responses of inducible skin-associated lymphoid tissue (iSALT) [4]. For example, genetic mutations of fillagrin, an important epidermal differentiation molecule, has been found in a significant proportion of patients with AD [5]. The aberrant immune responses in AD involve the regulation of T cells, antigen presentation cells, and cells in innate immunity among the iSALT [6].

In addition to genetic factors, environmental factors and immune adaptations to these factors may contribute importantly to the occurrence of AD as well as disease severity and disease course [2]. Little research has been undertaken to elucidate the regulatory mechanisms of skin immunity adaptation to the environmental stress [7]. However, aryl hydrocarbon receptor (AhR) and the aryl hydrocarbon receptor nuclear translocator (ARNT) axis are known to lie at the intersection of several crucial adaptive pathways in cells facing environmental stresses [8]. For example, when ligand binds to environmental chemicals such as dioxin (TCDD), AhR translocates into the nucleus and dimerizes with ARNT (AhR nuclear translocator), an important transcriptional factor [9]. AhR and ARNT are known to recognize many small xenobiotic and natural molecules and to play an role in the pathogenesis of many human diseases [10].

* Corresponding author at: National Environmental Health Research Center, No. 35, Keyan Road, Zhunan Town, Miaoli County 35053, Taiwan.
E-mail address: skhuang@nhri.org.tw (S.-K. Huang).
[1] Equal contribution.

http://dx.doi.org/10.1016/j.intimp.2016.04.017
1567-5769/

ARNT is required for tumor initiation by benzo[*a*]pyrene (BP) in mouse epidermis [11]. It might be possible that BP could bind to AhR to activate ARNT, which would lead to tumor formation. Our previous study reported an association between a lifetime accumulated risk to cigarette smoking and the development of AD [12]. The tobacco smoke contains many chemicals and polycyclic aromatic hydrocarbons (PAH) [13], a major portion being BP [14]. BP may induce aberrant immune responses [15] and the subsequent development of cancers [16]. Aside from its role in carcinogenesis, BP is able to bind with high specificity to the aryl hydrocarbon receptor (AhR), activating ARNT, modifying the expression of CYP1A1, and regulating inflammation [17]. Vigorous efforts have been made to study the role of AhR/ARNT axis in cancers initiated by BP [11,18]. However, little research attention has been paid to understand the mechanisms underlying apparent inflammatory and erythematous responses following epicutaneous exposure to BP. Because targeted K14-driven deletion of ARNT in the mouse epidermis has been reported to cause failure of epidermal barrier function [19], ARNT may be critical to homeostasis of skin barrier function, making it a potential candidate in AD. Roberston et al., using in vitro skin equivalents, showed that ARNT controls the expression of epidermal differentiation genes through HDAC- and EGFR-dependent pathways, possibly impairing barrier function leading to the development of AD [20]. However, it is unknown how AhR/ARNT axis regulates the epicutaneous sensitization process of AD. This study aims to determine the role of AhR/ARNT in the sensitization process of AD by animal studies, cell models, and human atopic skin examinations.

2. Materials and methods

2.1. Immunofluorescence (IF)

The specimens of skin from AD and controls were obtained from 8 patients (4 men and 4 women aged 21–48 years) and 6 controls (3 men and 3 women aged 25–57 years). All patients and control subjects provided informed consent before receiving biopsies. This study was approved by the Institutional Review Board of the affiliated hospital. For the animal study, mouse skin samples were obtained by 2-mm punch biopsy. The tissue sections from both humans or mice were fresh frozen, fixed in acetone (Sigma, St Louis, MO) at 20 °C for 5 min, air dried for 5 min, and washed in PBS for 15 min. They were incubated overnight at room temperature in a humidifier with or without the addition of a rabbit *anti*-AhR IgG (1:50, Santa Cruz Biotechnology, Dallas, TX), a rabbit *anti*-ARNT IgG (1:100, Cell Signaling, Danvers, MA), or a rabbit anti-CYP1A1 IgG (Novus Biologicals, Littleton, CO). The slides were washed in PBS, and then incubated with either Alexa Fluor® 488 -goat anti-rabbit IgG or Alexa Fluor® 568-goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) for 1 h at room temperature. Nuclei were counterstained with DAPI. Image analysis was performed by NIH Image J, an open image analysis software program (http://rsbweb.nih.gov/ij/) with a plugin that measures the fluorescent intensity score from 0 to 255. Numbers of positive-stained cells were measured and averaged in the epidermis from five random high power fields. Based on an artificial cut point in the intensity score, the percentages of AhR-, ARNT-, or CYP1A1-positive-staining epidermal DAPI-stained nucleated cells in the epidermis were then calculated. Similarly, the fluorescent intensity (relative unit from 0 to 255) of *E*-cadherin per keratinocytes from five random high power fields was also acquired for further statistical analysis.

2.2. Mice

Female C57BL mice (8–12 weeks old) were obtained from the National Laboratory Animal Center-Tainan Facility (Tainan, Taiwan). AhRd congenic mice, which have been described previously [21] and which are characterized as having a low affinity form of cytosolic Ah receptor, were obtained from Jackson Laboratory (Bar Harbor, ME). AhRd congenic mice (B6.D2N-Ahrd/J) carry a low-responder allele (AhRd), whereas C57BL/6 mice carry high-responder allele (AhRb-1) [21]. It has been demonstrated that the affinity of AhRd for ligand is 10–100 times lower that of AhRb alleles [22]. All mice were housed in a specific pathogen-free animal facility and treated following experimental protocols approved by the Animal Care and Use Committee of the Kaohsiung Medical University (Approval Number 96019). OVA (Grade V) was purchased from Sigma-Aldrich (St Louis, MO).

2.3. Ova-epicutaneous sensitization with patched BP

Mice were immunized as previously described [23]. Briefly, 20 ml of OVA (100 mg/ml) was placed on the disc of a Finn chamber (Epitest, Tuusula, Finland). This disk was applied to an area of shaved skin on the back of a mouse. For each course of immunization, freshly prepared OVA patches were applied on five consecutive days. The BP-treated group was patched with BP at 0.5 ppm/mice/day immediately prior to receiving the patch on days 1–5. Control mice received a similar treatment but patched with acetone.

2.4. Flow cytometry

Skin-draining LNs (axillary, subscapular, and inguinal) were excised 96 h after the start of the immunization course. LN cells were prepared by digestion of isolated LNs with 2.5 mg/ml collagenase for 30 min at 37 8C, and the CD11c + cells were isolated by *anti*-CD11c microbeads (Miltenyi, Bergisch Gladbach, Germany). Cells were stained with combinations of antibodies (CD11c-APC, CD207-PE) (both from e-Bioscience, San Diego, CA) and their isotype controls.

2.5. Cell counting and ELISA

Ten days after the start of the immunization course, mice were sacrificed, and axillary, subscapular, and inguinal LNs were harvested. Pooled LN cells (1×10^6) were cultured in the presence or absence of 100 mg/ml OVA. Cell proliferation was measured using an automatic counter (Countess, Invitrogen, Carlsbad, CA) according to manufacturer's directions. Supernatants were harvested 48 h later and stored at minus 80 °C. The levels of IL-5, IL-13, IL-10, TGF-β, and IL-17 were measured by standard sandwich ELISAs (R&D, Minneapolis, MN) with the limit of detection at 10 pg/ml.

2.6. Measurement of E-cadherin in human primary skin keratinocytes treated with AhR agonists and antagonists

Human primary cultured keratinocytes were obtained from adult foreskins as previously described [12]. Keratinocytes at early passage (typically <5) were then grown in keratinocyte-SFM medium (Gibco, Grand Island, NY) free of supplements 24 h before the experiments. Keratinocytes reaching 80–90% of confluence were treated with AhR ligands (ITE 5 μM or BP 10 μM) or AhR antagonists (CH223191 10 μM) for 24 h. The expression of *E*-cadherin was measured by immuno fluorescence as described above. The keratinocytes were incubated overnight at room temperature in a humidifier with or without the addition of a rabbit *anti*-*E*-cadherin IgG (1:100, Abcam, Cambridge, MA). The slides were washed in PBS, and then incubated with Alexa Fluor® 568-goat *anti*-rabbit IgG (Invitrogen, Carlsbad, CA) for 1 h at room temperature. Nuclei were counterstained with DAPI.

2.7. Statistical analyses

The numeric variables between two groups were compared by non-parametric Mann-Whitney *U* test. These numeric variables include the