CELLULAR ANTENNA IN SKIN: PLIMARY CILIA AS INFLAMATORY SKIN DISEASE MARKER.

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SUMMARY

Background:

The skin is the largest immune organ facing the outside of the body. The skin exerts an immune responses to maintain homeostasis when stimulated with viruses, pathogens, air pollutants, also chemical materials including cosmetics. This immune function is indispensable for skin homeostasis, however excessive immune responses cause the overproduction of cytokines and unbalanced cell differentiation / proliferation, which may cause skin diseases. Therefore, it is widely desired to elucidate the regulation mechanism(s) of the skin immunity, also to establish the method how to analyze skin inflammation quantitatively by using various indexes.

Methods:

We observed primary cilia, known as ‘cellular antenna’ to sense extracellular environment, in human skin. Also, we investigated the primary cilia functions in DC proliferation and maturation by using in vitro cell culture system.

Results:

We confirmed that only langerhans cells (LCs) and keratinocytes have primary cilia in human epidermis. In atopic dermatitis epidermis, percentage of ciliated cells were significantly increased. Disassemble of primary cilia in DCs in vitro decreased proliferation marker Ki67, whereas increased maturation marker CCR7.

Conclusion:

Our results suggested that primary cilia in LCs promoted proliferation while suppressing maturation, suggesting the physiological function of primary cilia in maintaining homeostasis of skin immunity.

Keyword; inflammatory skin; langerhans cells; keratinocytes; primary cilia.

Introduction

Skin is the biggest tissue playing immunologically important roles in homeostasis to prevent infections. Skin is composed of three layers; epidermis, dermis, and subcutaneous tissue, and most outer layer epidermis mainly contains keratinocytes (KCs) which finally form the stratum corneum working as a physical barrier against pathogens (1). Immune cells, including langerhans cells (LCs), which have a similar role as dendritic cells (DCs), also exist in epidermis and maintain skin homeostasis by working as antigen-presenting cells to activate T cells (2). When LCs incorporate microbial antigens, they immediately migrate toward the lymph node and activate T cells (2). It has long been accepted that LCs have a pivotal role in bridging innate and acquired immunity, which is required for skin homeostasis. In contrast to their contribution in maintaining homeostasis, they are also involved in the pathology of allergic skin diseases, such as atopic dermatitis (AD)(3). Therefore, understanding of molecular mechanisms how LCs and KCs involved in pathogenesis is required.

The prevalence of AD has increased greatly in the past 30 years, and it is widely known that environmental factors such as mite antigen in house dust can trigger allergic disease. In both the early and chronic stages of AD, type 2 immune responses, which are characterized as the elevation of several type 2 cytokines and IgE production, are dominant (4). AD often features skin barrier disruption, leading to dryness, itchiness, and invasion by pathogens such as *Staphylococcus aureus*. Topical steroids, tacrolimus, and moisturizers are employed in its treatment, but because of its complicated pathogenesis, it often recurs after improvement. An important finding reported that proliferating LCs were significantly increased in AD patients (5) and suggested that they promoted Th2-dominant condition. As such, it is clinically important to regulate both inflammation and skin barrier maintenance by targeting both KCs and LCs, however, how they are regulated is not well understood.

Primary cilia are unique organelles protruding into extracellular spaces from a basal body and works as a platform for signaling pathways (Fig. 1). Intraflagellar transport (IFT) system is essential for axonome elongation and ciliary protein transport. *IFT* gene mutations or disruption of this transport system eliminate primary cilia, resulting in developmental defects, signaling defects and ciliopathy (6). Primary cilia are formed when cells are in G0 or G1 cell cycle phase, and components of primary cilia regulate cell cycle progression. Thus, primary cilia formation and the cell cycle tightly regulate each other, and widely thought that primary cilia regulate cell proliferation and differentiation in many types of cells or tissues. Several researches suggested the physiological functions of primary cilia in adult tissues, however the precise roles of them in adult skin/immune system have not been explored until recently. We therefore examined primary cilia in

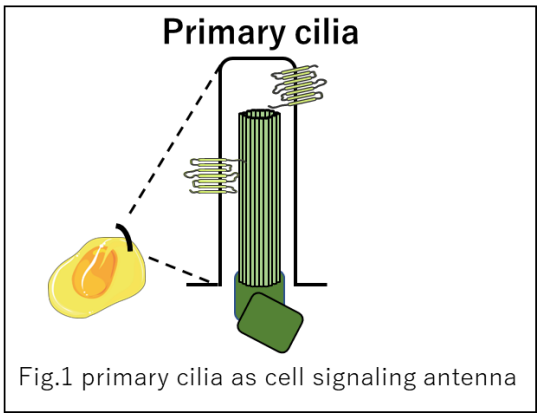

Fig.1 primary cilia as cell signaling antenna

human adult epidermis, and we found the significant increase of primary cilia in atopic dermatitis epidermis with increasing proliferating cells. As our *in vitro* experiments using primary blood monocytes, DCs and THP1 cell lines suggested the primary cilia function in cell proliferation, our finding suggests the primary cilium function in physiological relevance in skin homeostasis and pathological condition of the skin.

Materials and Methods

Human skin biopsy samples

Biopsy specimens were obtained from 15 adult (over 20 years of age) patients diagnosed as AD and from 5 healthy donors. All donors provided written informed consent prior to sampling according to the Declaration of Helsinki. This study was approved by the institutional ethics committees of Nagoya City University Graduate School of Medical Sciences and of Osaka University. Approved protocol number is; 60-18-0003, and 薬人 2019-1, respectively.

Isolating human monocytes and DCs

Ethical review for medical and health research involving human subjects were approved by Osaka university (Approval number; 薬人 2019-1) and by Nagoya City University (Approval Number: 60-18-0003). All experiments were followed by ethical guidelines of facilities. Whole peripheral blood from 16 year-old to 69 year-old healthy volunteers which was not fit for blood products was purchased from the Japanese Red Cross Society according to the Guidelines on the Use of Donated Blood in R&D, etc. We obtained totally 164 healthy blood from Japanese Red Cross Society (JRCS) for PBMC cultures and performed each experiment. Peripheral blood mononuclear cells (PBMCs) were isolated from the whole peripheral blood using Ficoll-Paque PLUS (GE Healthcare) and density-gradient centrifugation. To lyse erythrocytes, they were incubated with ACK buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 15 minutes at room temperature. Dead cells were removed using a Dead Cell Removal Kit (Miltenyi Biotec, 130-042-401). The isolated PBMCs were labeled with CD14 microbeads (Miltenyi Biotec, 130-050-201), and a CD14-positive cell population was isolated using magnetic separation, which we considered to be monocytes. DCs were isolated with a Blood Dendritic Cell Isolation Kit II, human (130-091-379, Miltenyi Biotec), from the negative fraction of the CD14-positive fraction.

Immunostaining

Paraffin-embedded human skin tissue was deparaffinized in xylene 2 times for 10 min each. The samples were then immersed in 100% ethanol, 95% ethanol, and 2 lots of deionized water for 10 min each. After rehydration, antigens were retrieved: The samples were immersed in 1 mM EDTA in

deionized water and boiled by using a microwave for 15 min. Samples were then incubated with blocking buffer (10% FBS, 0.1% Triton X-100 in PBS) at room temperature for 1 h. PBMC, Human primary DCs isolated by MACS / FACS sorting and T cells/NK cells/B cells isolated by FACS were fixed by 4% PFA for 30 min at room temperature. Cells were mounted on MAS-coated slide glass (Matsunami) and we proceeded to the next step. Cells were incubated with blocking buffer (10% FBS, 0.1% Triton X-100 in PBS) at room temperature for 1 h. After blocking and permeabilization, we proceeded to primary antibody incubation for all samples.

First antibodies (listed below) were diluted in PBS. The details of the antibodies are as follow; anti-acetylated α-tubulin (1/1000 dilution, T7451, clone 6-11B-1, Sigma), anti-langerin (1/1000 dilution, ab192027, clone EPR15863, Abcam), anti-vimentin (1/1000 dilution, ab92547, clone EPR3776), anti-ki67 (1/1000 dilution, #9449, clone 8D5, Cell Signaling Technology; 1/1000 dilution, ab16667, clone SP6, Abcam), anti-K10 (1/1000 dilution, PRB-159P, covance), anti-K14 (1/1000 dilution, ab7800, clone LL002, abcam), anti-CCR7 (1/100 dilution, MAB197, clone 150503, R&D Systems), anti-pericentrin (1/1000 dilution, A301-348A, Bethyl), and anti-GFP (1/1000 dilution, SC-9996, clone B-2, Santa Cruz Biotechnology).

All antibodies were incubated at 4 °C overnight. After washing with wash buffer (0.1% Tween-20 in PBS) by 3 times, incubation with secondary antibodies (Alexa 488-conjugated IgG, Alexa 594-conjugated IgG, both were from Thermo Fisher Scientific) containing Hoechst 33342 (Thermo Fisher Scientific) were performed at room temperature for 2 h. After rinsing in wash buffer, samples were mounted with ProLong Gold antifade reagent (Thermo Fisher Scientific). Immunofluorescence images were captured by using Olympus confocal microscope FV1200 (Olympus) equipped with100 x objective, or Carl Zeiss LSM700/LSM710 confocal microscope (Carl Zeiss) equipped with 100 x or 60 x objective. The number of ciliated cells was counted and statistically analyzed using the GraphPad Prism 7 software.

Results

To examine the primary cilia distribution in human adult skin, we took healthy human skin samples and visualized the primary cilia in epidermis and dermis by staining them with acetylated tubulin using fluorescent confocal microscopy. As expected, we found many ciliated cells in dermis which suggests that most ciliated cells were fibroblasts. We also found primary cilia in the K14 positive epidermal basal layer where proliferating keratinocyte stem cells are populous. Primary cilia-like structures were also detected in the K10 positive stratum spinosum and granular layer at a lower frequency than in the basal layer. We next determined the type of ciliated cell by immunostaining with LCs marker langerin, and T cell marker CD4 or CD8. We found that only langerin positive LCs were positively merged with acetylated tubulin. Since LCs are known as dendritic cells (DCs) in epidermis, we then investigated the primary cilia functions in DCs derived from human peripherial blood. We stimulated primary DCs with

strong Th1 responses inducers including TNFα and Prostaglandin E2 (PGE2) for 24 h, respectively, and we observed that both of TNFα and PGE2 significantly decreased primary cilia formation in a dose-dependent manner (Fig. 2). To examine if Th2 cytokines also regulate primary cilia assemble, we stimulated DCs with IL-4 and GM-CSF, and we observed the significant increase of ciliated cells in GM-CSF treated group with increasing cell proliferation (Fig. 2).

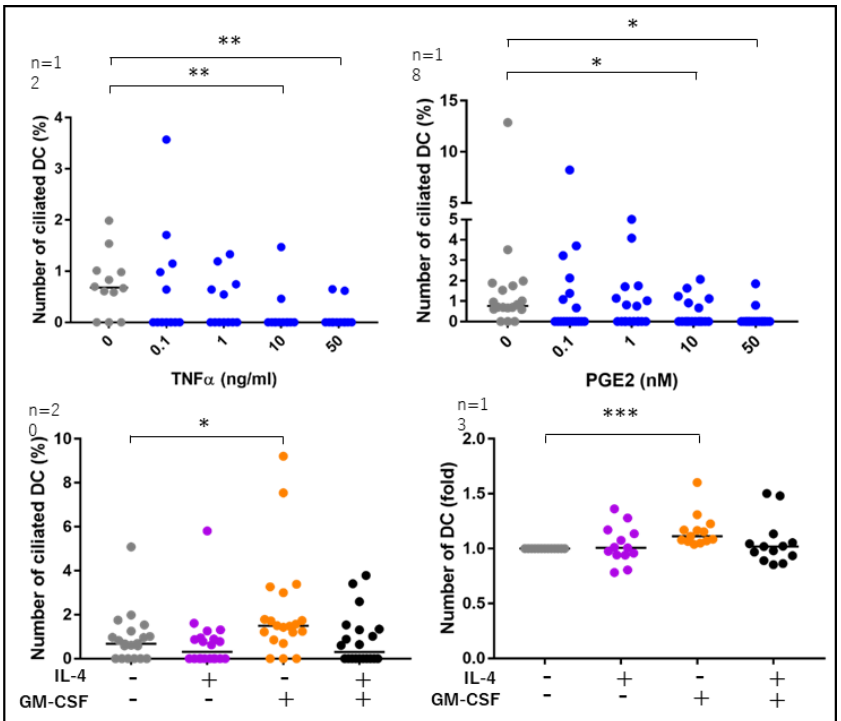

Fig.2 Th2 cytokine GM-CSF promotes ciliogenesis while Th1 cytokines inhibits it.

To further examine the function of primary cilia in DCs, we performed knockdown experiment targeting IFT88, which is indispensable for primary cilia assembly. Knockdown of *IFT88* increased DC maturation markers, including Cluster of Differentiation 86 (CD86) and C-C chemokine receptor type 7 (CCR7) in THP1-derived immature DCs (Fig. 3), while Ki67 expression was downregulated (Fig. 3).

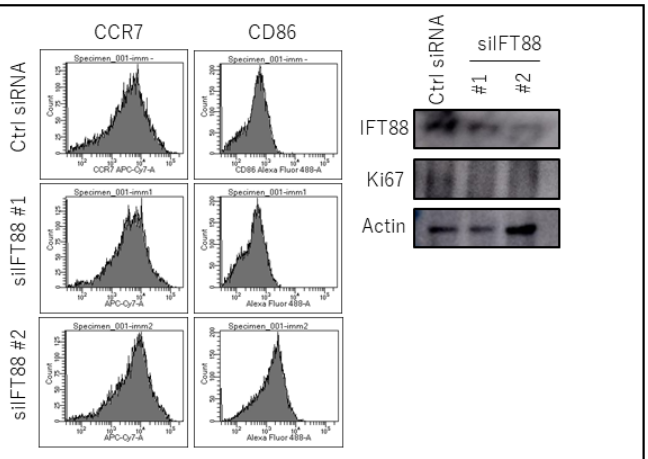

Fig.3 Disassemble of primary cilia upregulated DC maturation marker while decreasing proliferation marker ki67

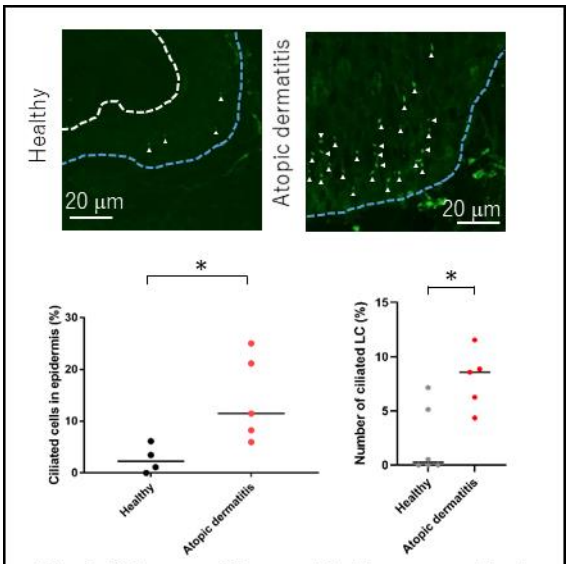

Fig.4 Primary cilia are highly assembled in atopic epidermis

LCs and KCs proliferate extensively in epidermis with AD (5). To address whether atopic condition alters primary cilia formation in epidermal cells, we investigated the frequency of primary cilia in human epidermis with AD. We observed that percentage of ciliated LCs/KCs and proliferating LCs/KCs were significantly increased in atopic dermatitis (Fig. 4). In summary, immunostaining results in human

atopic dermatitis skin suggested excessive proliferation and abnormal primary cilia formation in AD, which in turn caused hyperproliferation and sustenance of immature LCs and KCs in AD.

Discussion

We identified primary cilia in human epidermis and immune cells from blood (Fig. 1), and their formation in epidermis were highly promoted in AD (Fig. 4). We found that Ki67-positive ciliated cells were highly increased in AD epidermis (Fig. 4), so we hypothesized that there is a relationship between primary cilia formation and epidermal cell proliferation in AD. Primary cilia formation are basically inhibited in G2/M and S phases, and this phenotype was not found in healthy epidermis (Fig. 4G). Our finding raised the possibility that cell cycle and proliferation may be disrupted in AD, which might be a pathophysiological phenotype of the disease. We have not identified what induces AD directly, however, our data proposes that Th2 cytokine GM-CSF and mite antigen working as allergen, *Df*, are candidates to develop AD by regulating primary cilia formation and proliferation of epidermal cells. Interestingly, prevalence of AD or asthma in ciliopathy patients, along with other phenotypes caused by primary cilia defect, are higher than normal cohort (7)(8). Furthermore, an interesting report about the relationship between Bardet–Biedl Syndrome, a ciliopathy caused by primary cilia dysfunction, and autoimmune diseases development has been published recently (9). These finding still has an enigmatic question of why ciliopathy patients highly developed autoimmune diseases. Although future investigations are required to answer this, our finding here propose the mechanism of atopic dermatitis and therapeutic potential of inflammatory skin diseases in human.

Conclusion

1. Primary cilia is over-assembled in atopic dermatitis epidermis.
2. Th1 cytokines significantly inhibited primary cilia formation in DCs/KCs while Th2 cytokines promoted assembling.
3. Disassemble of primary cilia induced cell maturation.

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Conflict of Interest Statement.

This study was based on joint research with Mandom corporation. My co-authors Fujita and Okada are employee of Mandom corporation.

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