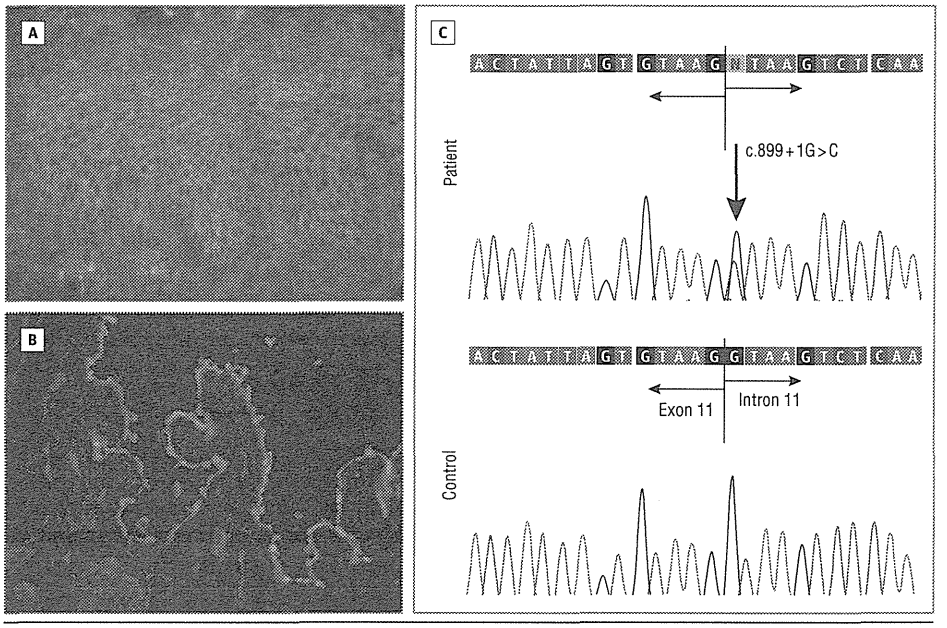
Figure 2. The Results of Immunologic and Genetic Studies for Case 1



A and B, The results of IgG indirect immunofluorescence studies of monkey esophagus (A) and rat bladder (B) (original magnification ×200 for both). C, The results of *ATP2C1* gene analysis of genomic DNA for case 1 (top) and healthy control individual (bottom), showing a heterozygous G>C transition at first residue in intron 11 only in the patient (down-pointing arrow).

younger, as they aged into their 60s, they manifested atypical clinical features and unique disease courses. Intriguingly, the clinical features in the 2 cases were significantly different from each other, in spite of the presence of the same genomic mutation. Taking the changes in clinical features during the disease courses of the 2 cases together, we speculate that some environmental conditions, in addition to the genetic defect, may affect the development of skin lesions of HHD.

A study of 58 patients with HHD in the United Kingdom[2] indicated that predilection sites were the flexure areas, including the groin, axilla, perineum, inframammary region, umbilicus, and retroauricular region, in descending order, as well as neck/nape, shoulders, chest, arms, and back.[2] The abdomen was not involved in any cases. Only a few cases showed lesions on the popliteal fossae, head, and face, which were frequently associated with eczematous changes but rarely showed bullae or pustules.

Two clinically unique and atypical HHD cases have been reported,[4,5] although genetic study was not performed in either case. One case showed seborrheic dermatitis-like lesions on the scalp as well as other typical HHD lesions,[4] while the other case showed generalized vesiculobullous lesions with occasional pustules.[5] To our knowledge, our patients represent the first sibling cases with seborrheic dermatitis-like, pemphigus vulgaris-like, or pemphigus foliaceus-like atypical clinical and histopathologic features of HHD diagnosed by genetic analysis.

Shigeruko Iijima, MD
Takahiro Hamada, MD
Mirei Kanzaki, MD
Chika Ohata, MD
Takashi Hashimoto, MD

Author Affiliations: Department of Dermatology, Mito Saiseikai General Hospital, Mito, Ibaraki, Japan (Iijima, Kanzaki); Department of Dermatology, Kurume University School of Medicine, and Kurume University Institute of Cutaneous Cell Biology, Kurume, Fukuoka, Japan (Hamada, Ohata, Hashimoto).

Corresponding Author: Takashi Hashimoto, MD, Department of Dermatology, Kurume University School of Medicine and Kurume University Institute of Cutaneous Cell Biology, 67 Asahimachi, Kurume, Fukuoka 830-0011, Japan (hashimot@med.kurume-u.ac.jp).

Published Online: December 18, 2013. doi:10.1001/jamadermatol.2013.5666.

Conflicts of Interest Disclosures: None reported.

Funding/Support: This study was supported by Grants-in-Aid for Scientific Research Nos. 20390308, 20591331, 21659271, 23591634, 23791298, 23791299, 23791300, 23791301, 24659534, 24591672, 24591640, 24791185, and 22590543 and by Supported Program for the Strategic Research Foundation at Private Universities 2011-2015 from the Ministry of Education, Culture, Sports, Science and Technology; and by Research on Measures for Intractable Diseases Project matching fund subsidies "H23-028 (K. Iwatsuki)" and "H24-038 (T. Hamada)" from the Ministry of Health, Labor and Welfare. The study was also supported by grants from the Kaibara Morikazu Medical Science Promotion Foundation, Ishibashi Foundation, Kanae Foundation for the Promotion of Medical Science, Takeda Science Foundation, Chuo Mitsui Trust and Banking Company, Limited, and Nakatomi Foundation.

Role of the Sponsor: The sponsors had no role in the design and conduct of the study; collection, management, analysis, or interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

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DOI: 10.1111/exd.12418
www.wileyonlinelibrary.com/journal/EXD

Distinct protein expression and activity of transglutaminases found in different epidermal tumors

Tadashi Karashima[1], Minao Furumura[1], Norito Ishii[1], Bungo Ohyama[1], Hiroshi Saruta[1], Yohei Natsuaki[1], Takekuni Nakama[1], Chika Ohata[1], Daisuke Tsuruta[1]*, Kiyotaka Hitomi[2] and Takashi Hashimoto[1]

[1]Department of Dermatology, Kurume University School of Medicine, and Kurume University Institute of Cutaneous Cell Biology, Fukuoka, Japan and [2]Department of Applied Molecular Biosciences, Graduate School of Pharmaceutical Sciences, Nagoya University, Chikusa, Nagoya, Japan
Correspondence: Takashi Hashimoto, Department of Dermatology, Kurume University School of Medicine, and Kurume University Institute of Cutaneous Cell Biology, 67 Asahimachi, Kurume, Fukuoka 830-0011, Japan, Tel.: +81-942-31-7571, Fax: +81-942-34-2620, e-mail: hashimot@med.kurume-u.ac.jp
**Presentaddress:* Department of Dermatology, Osaka City University Graduate School of Medicine, Osaka, Japan

Abstract: We investigated protein expression and *in situ* activity of transglutaminases (TGs) in normal skin and various epidermal neoplasms. In normal skin, TG1 protein expression and TG activity were found at keratinocyte cell membranes in upper epidermis and granular layer, respectively. In seborrhoeic keratosis, TG1 protein was expressed evenly throughout tumors, while TG activity increased in gradient fashion from lower tumor area to cornified layer. In squamous cell carcinoma, TG1 protein was expressed at inner side of cell membranes, whereas TG activity was found in cytoplasm predominantly at horn pearls. In basal cell carcinoma, weak TG activity was found in cytoplasm of all tumor cells without the presence of TG1 protein. Immunoblotting and *in situ* activity assays using specific substrate peptides confirmed that TG2, but not TG1, contributed to the TG activity. These results suggested that different expression and activation of TGs may contribute to characteristics of the skin tumors.

Key words: basal cell carcinoma – keratinocyte differentiation – squamous cell carcinoma – transglutaminase

Accepted for publication 16 April 2014

Background

Transglutaminases (TGs) catalyze cross-linking reactions between proteins. During epidermal differentiation, epidermal TGs (TG1, TG3 and TG5) are involved in cornified envelope formation by cross-linking structural proteins (1,2). Among them, TG1 is considered to be crucial, because its knockout mice showed severely impaired epidermal barrier (3). In addition, expression patterns of epidermal TGs were related to tumor differentiation in squamous cell carcinoma (SCC) (4), confirming essential role of TGs in keratinocyte differentiation, which was suggested by previous

studies (5–8). On the other hand, TG dysfunction leads to tumor formation in various carcinomas (9,10).

In contrast to the epidermal TGs, TG2 (tissue TG) is ubiquitously expressed and involved in various biological events (11–13). The analysis of TG2 knockout mice showed impaired phagocytosis of apoptotic cell material and aberrant wound healing (14). In the skin, TG2 is primarily and variably expressed in epidermal basal layer to stabilize dermo-epidermal junction during tissue remodelling/repair (15,16). Although TG2 was expressed in highly invasive melanomas and breast cancers (17–20), TG2 could alternate extracellular matrix components by isopeptide cross-links and inhibit tumor invasion (21).

However, expression of functional TGs in epidermal neoplasms has not been investigated in detail. In this study, we investigated protein expression and *in situ* activity of TGs in normal skin and various epidermal neoplasms.

Questions addressed

The question was how TGs are involved in epidermal differentiation and human epidermal tumor formation.

Experimental design

Protein expression and activity of TGs were studied for three normal human skins and five samples each of the cutaneous tumors seborrhoeic keratosis, SCC and basal cell carcinoma (BCC). All tumors were excised from patients with no particular complications and had typical clinical and histopathological features. Detailed protocols for all methods are described in Supporting Information, Data S1. The results were generally the same among 5 samples of each tumor.

Results

Normal skin

In normal human skin, immunofluorescence revealed that TG1 protein was expressed at keratinocyte cell membranes in cornified, granular and middle-upper spinous layers (Fig. 1a,c). TG was activated at keratinocyte cell membranes in granular layer with weak activity in upper epidermis (Fig. 1b,c). No TG activity was observed in either cornified or basal layer. Expression pattern of involucrin was nearly identical to that of TG1 (Fig. 1d).

Seborrhoeic keratosis

In seborrhoeic keratosis, TG1 protein was expressed at cell membranes in entire tumor areas (Fig. 1e,g). In contrast, TG was activated strongly in cornified and subcorneal layers but much weakly in tumor cells, forming gradient from lower tumor areas to cornified layer (Fig. 1f,g). Involucrin was expressed in almost similar pattern to TG activity (Fig. 1h).

Scc

In well-differentiated SCC, most prominent TG1 protein expression and TG activity were observed at horn pearls in tumor areas, although slightly different distribution was noted (Fig. 1i–k). However, interestingly, distribution patterns were different between TG1 protein expression and TG activity. Thus, TG1 protein was expressed at cell membranes of tumor cells, while TG activity was present only in cytoplasm (Fig. 1l). There was no difference in TG activity between pH 7.4 and pH 8.4 (data not shown).

Bcc

BCC tumor cells expressed neither TG1 protein nor involucrin, while both proteins were strongly expressed in overlying epidermis (Fig. 1m,p). TG activity was detected in cytoplasm of BCC tumor cells, although it was weaker than that in overlying epidermis (Fig. 1n,o).

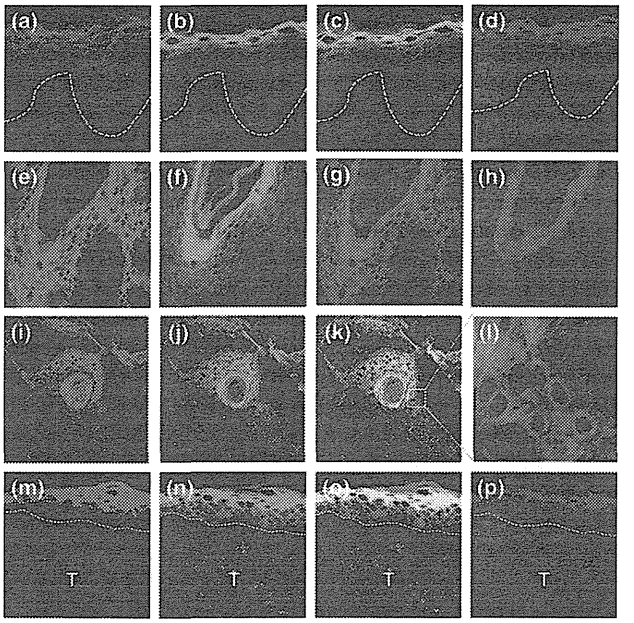

Figure 1. Expression of TG1 protein, *in situ* TG activity and expression of involucrin protein. Normal human skin (a–d). Seborrhoeic keratosis (e–h). SCC (i–l). BCC (m–p). TG1 protein expression (a, e, i, m). TG activity (b, f, j, n). Merged images of TG activity and TG1 protein expression (c, g, k, l, o). Involucrin protein expression (d, h, p). (l) Higher magnification view of area indicated by square in panel k. Broken lines indicate basement membrane zone. 'T' indicates tumor nests of BCC.

Detection of expression of TG2 in BCC tumor cells

To clarify the discrepancy between TG1 protein expression and TG activity in BCC, we first analysed expression of TG1 and TG2 by immunoblotting using samples from BCC, as well as normal human epidermal extracts, whole skin extracts, seborrhoeic keratosis, SCC and normal human fibroblasts (NHFs) (Fig. 2a,b). TG1 protein was detected in epidermal extracts, whole skin extracts, seborrhoeic keratosis and SCC, but not in BCC and NHFs (Fig. 2a). In contrast, TG2 protein was detected in BCC, NHFs, epidermal extracts and whole skin extracts, but not in seborrhoeic keratosis and SCC (Fig. 2b).

By immunohistochemistry, we confirmed expression of TG2 at cell membranes and in cytoplasm of BCC tumor cells (Fig. 2c). In normal skin, TG2 was consistently expressed in endothelial cells and fibroblasts in dermis, and in epidermal basal keratinocytes in a sporadic fashion (Fig. 2d).

In situ activity assays specific for TG1 or TG2 in BCC

To examine whether TG2 is functional in BCC, we performed *in situ* activity assays using FITC-labelled peptides K5 and T26, which are specific substrates for TG1 and TG2, respectively (22,23). In BCC, TG1 activity was seen in overlying epidermis, but not in BCC tumor cells (Fig. 2e). In contrast, TG2 activity was observed clearly in BCC tumor cells (Fig. 2f). Weak activity of TG2 was also seen in lower epidermis, as well as fibroblasts and endothelial cells in the dermis (Fig. 2f). No specific reactivity was seen by immunofluorescence using negative control peptides (K5QN and T26QN) (data not shown).

Conclusions

In this study, TG1 protein and involucrin were expressed in granular layer of epidermis of normal skin, confirming the results in

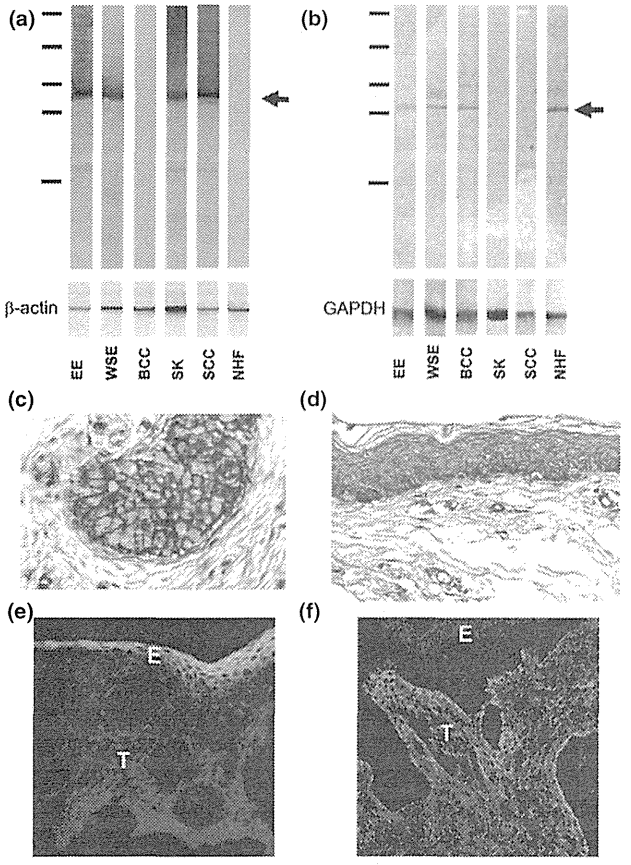

Figure 2. The results of immunoblotting and immunohistochemistry for TGs and *in situ* activity assays for TG1 and TG2 in BCC. (a, b) The results of immunoblotting for TG1 (a) and TG2 (b). EE: epidermal extracts. WSE: whole skin extracts. SK: seborrhoeic keratosis. NHF: extracts of NHFs. Bars in the left of each panel indicate molecular weight markers (from top to bottom, 200 kDa, 150 kDa, 100 kDa, 75 kDa and 50 kDa). Arrows indicate protein bands for TG1 (a) and TG2 (b). As loading controls, β-actin (a) and GAPDH (b) were used. (c, d) Immunohistochemical staining for TG2 in BCC (c) and in normal skin (d). TG2 expression was shown as dark blue colour. Nuclear counterstaining was done with nuclear red. (e, f) *In situ* activity assays of TG1 and TG2 in BCC. (e) Incorporation of FITC-labelled peptide (K5) specific for TG1. (f) Incorporation of FITC-labelled peptide (T26) specific for TG2. Nuclei are stained with DAPI (blue). 'E' indicates epidermis. 'T' indicates tumor nests of BCC.

previous study (24,25). Expression of TG1 protein and involucrin and *in situ* TG activity in seborrhoeic keratosis were almost identical to those in normal skin. In contrast, in SCC tumor cells, TG1 protein was expressed at cell membranes, while TG activity was seen in cytoplasm. In BCC, while TG1 protein was not expressed as shown in previous study (4), weak TG activity was observed.

The different patterns seen in SCC; that is, membrane-associated TG1 protein expression and cytoplasmic TG activity were probably due to suppression of activity of membranous TG1 (23,26,27).

Most intriguingly, we found that functional TG2 expression was responsible for the TG activity in BCC. TG2 expression may play a role in long-term benign course and non-metastatic nature in BCC, as TG2 is known to inhibit tumor invasion and induce tumor cell apoptosis (21).

In conclusion, protein expression of TGs and involucrin and *in situ* TG activity were found differently in various epidermal tumors. In particular, TG2 was expressed and functional in BCC tumor cells. These results suggested that different expression and activation of TG may contribute to specific characteristics in the skin tumors.

Acknowledgements

We gratefully appreciate the technical assistance of Ms. Ayumi Suzuki and the secretarial work of Ms. Tomoko Tashima, Ms. Mami Nishida and Ms. Shoko Nakamura. We thank the patients for their participation. This study was supported by Grants-in-Aid for Scientific Research (No. 20390308, 20591331, 21659271, 23591634, 23791298, 23791299, 23791300, 23791301, 24659534, 24591672, 24591640, 24791185), and supported programme for the Strategic Research Foundation at Private Universities from the Ministry of Education, Culture, Sports, Science and Technology; and by 'Research on Measures for Intractable Diseases' Project: matching fund subsidy (H23-028 to K. Iwatsuki, and H24-038 to T. Hashimoto) from the Ministry of Health, Labour and Welfare. The study was also supported by grants from the Kaibara Morikazu Medical Science Promotion Foundation, Ishibashi Foundation, Kanae Foundation for the Promotion of Medical Science, Takeda Science Foundation, Chuo Mitsui Trust and Banking Company, Limited, and Nakatomi Foundation.

Author contribution

TK, MF, NI, BO performed the experiments. TK, MF, CO, DT, TH prepared the manuscript. HS, YN, KH contributed essential reagents and samples. TK, TH originally designed this study. TK, MF, KH, TH revised the manuscript.

Conflict of interest

The authors have declared no conflicting interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Materials and methods.

THE JOURNAL OF DERMATOLOGY

doi: 10.1111/1346-8138.12472

Journal of Dermatology 2014; **41**: 386–392

ORIGINAL ARTICLE

Mouse bone marrow-derived dendritic cells can phagocytize the *Sporothrix schenckii*, and mature and activate the immune response by secreting interleukin-12 and presenting antigens to T lymphocytes

Masahiro KUSUHARA,[1,2,*] Hua QIAN,[1,*] Xiaoguang LI,[1,*] Daisuke TSURUTA,[1,3] Atsunari TSUCHISAKA,[1] Norito ISHII,[1] Chika OHATA,[1] Minao FURUMURA,[1] Takashi HASHIMOTO[1]

[1]*Department of Dermatology, Kurume University School of Medicine, and Kurume University Institute of Cutaneous Cell Biology,* [2]*Kusuhara Dermatology Clinic, Fukuoka, and* [3]*Department of Dermatology, Osaka City University Graduate School of Medicine, Osaka, Japan*

ABSTRACT

In sporotrichosis, dermal dendritic cells were considered to participate in induction of the immune responses against *Sporothrix schenckii* infection. However, it is still unclear whether and how dermal dendritic cells were involved in the progress. To clarify the pathogenic role of dermal dendritic cells (DC) in sporotrichosis, we examined the phagocytosis, maturation stages, cytokine production and antigen-presenting ability of mouse bone marrow-derived DC after stimulation with *S. schenckii*. By analysis of flow cytometry, electron microscope and confocal microscope, mouse bone marrow-derived DC were proved to be able to phagocytize the *S. schenckii*. The increased expression of CD40, CD80 and CD86 on the surface of *S. schenckii*-pulsed mouse bone marrow-derived DC was detected by flow cytometer, indicating that the *S. schenckii*-pulsed mouse bone marrow-derived DC underwent the maturation program. The secretory enhancement of interleukin (IL)-12, but not IL-4, was found in *S. schenckii*-pulsed mouse bone marrow-derived DC, suggesting the possible activation of T-helper 1 prone immune responses. Furthermore, *S. schenckii*-pulsed mouse bone marrow-derived DC were demonstrated to be capable of inducing the proliferation of T lymphocytes from BALB/c mice that were pre-sensitized with *S. schenckii*. Together, all the results implied that dermal DC may participate in the induction of immune responses against *S. schenckii* infection in sporotrichosis.

Key words: antigen presentation, bone marrow-derived dendritic cells, interleukin-12, phagocytosis, sporotrichosis.

INTRODUCTION

Sporotrichosis is a dermal, subcutaneous or rarely systemic fungal infection caused by dimorphic soil fungus, *Sporothrix schenckii*. Cutaneous and lymphocutaneous types of sporotrichosis are the most common clinical manifestations of *S. schenckii* infection.[1] In sporotrichosis, skin lesion generally appears as a papule, slowly enlarges, and becomes a nodule or ulcer. The nodular lesion later disseminates to proximal areas along lymph vessel streams.[2] Dissemination to visceral, osteoarticular, meningeal and pulmonary tissues occasionally occurs in immune-compromised hosts.[1] Histopathologically, sporotrichosis shows mixed granulomatous and pyogenic inflammation.[1] Until now, the immune response against *S. schenckii* infection has not been well elucidated.

Previous studies on cutaneous sporotrichosis suggested that $CD4^+$ T cells and macrophages play a pathogenic role in development of granuloma formation, and that a dendritic cell (DC) population was also observed in the granulomas.[3–5]

Dendritic cells are present in all peripheral tissues and accumulate at the sites of pathogen entry. DC are also specialized in linking innate and adaptive immune responses. They can take up pathogens in peripheral tissues, develop particular maturation programs selectively in response to different pathogens, migrate to lymphoid organs, and present antigens to T lymphocytes to initiate antigen-specific immune responses.[6]

Correspondence: Takashi Hashimoto, M.D., Department of Dermatology, Kurume University School of Medicine, and Kurume University Institute of Cutaneous Cell Biology, 67 Asahimachi, Kurume, Fukuoka 830-0011, Japan. Email: hashimot@med.kurume-u.ac.jp

*These authors contributed equally to this study.

Received 18 October 2013; accepted 18 February 2014.

Fungi can be recognized by DC through pattern recognition receptors including Toll-like receptors and lectin. Therefore, the dermal DC may also play important roles in inducing the immune response against *S. schenckii* in sporotrichosis.[7,8] We therefore hypothesized that DC may phagocytize *S. schenckii*, migrate into regional lymph nodes, and then present fungus antigens toward T lymphocytes to stimulate the *S. schenckii*-specific immune responses.

In the present study, the possible role of DC in inducing immune response to *S. schenckii* was investigated by using an *in vitro* co-cultured system, in which mouse bone marrow-derived DC (BM-DC) were co-cultured with *S. schenckii*. By this experimental system, we attempted to answer the following questions: (i) whether BM-DC are able to phagocytize *S. schenckii*; (ii) whether *S. schenckii*-pulsed BM-DC develop maturation; and (iii) whether *S. schenckii*-pulsed BM-DC can present antigens to *S. schenckii*-primed lymphocytes.

METHODS

Animals, BM-DC and fungi

Female BALB/c mice (aged 6–8 weeks) were purchased from CLEA Japan (Tokyo, Japan). Animal experiments were performed in the animal experiment center of Kurume University, in accordance with the institutional ethical guidelines. Bone marrow cells were isolated by aspiration from the mice, and cultured in complete RPMI-1640 medium containing 10% fetal bovine serum, 2 mmol/L glutamine, 50 μmol/L 2-mercaptoethanol, 100 μg/mL streptomycin sulfate and 100 U/mL penicillin. To induce the BM-DC, the bone marrow cells were stimulated with 20 ng/mL recombinant mouse granulocyte macrophage colony-stimulating factor (GM-CSF; GENZYME TECHNE, Minneapolis, MN, USA) and 10 ng/mL interleukin (IL)-4 (R&D Systems, Minneapolis, MN, USA), as described previously.[9] After 7 days of culture, non-adherent cells were collected as BM-DC. *S. schenckii* was isolated from patient skin lesions on the leg, and cultured on brain–heart infusion agar (Laboratories CONDA, Madrid, Spain) at 37°C to induce the yeast form of *S. schenckii*.[8] The yeast form of *Candida albicans*, which was also isolated from patient skin lesions of the genital area, was culture on Sabouraud's glucose agar to *S. schenckii* as control fungus. In addition, BM-DC were pretreated with 0.5 μg/mL polymyxin B for 4 h in related experiments to clear possible lipopolysaccharide (LPS) contamination.

BM-DC phagocytosis of *S. schenckii*

To observe the BM-DC phagocytosis of *S. schenckii*, immature BM-DC (1×10^6 cells) were stained with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse I-A/I-E (major histocompatibility complex [MHC] class II) monoclonal antibody (BD Biosciences, Sparkes, MD, USA) and yeast cells of *S. schenckii* (1×10^6 cells) were stained with CMTPX, a red fluorescence cell tracer (Molecular Probes, Eugene, OR, USA), which were subsequently mixed together and incubated at 37°C with 5% CO_2 for 0, 15, 30 and 60 min, respectively. For staining fungi, *S. schenckii* were incubated with 5 μmol/L CMTPX for 30 min, washed twice with phosphate-buffered saline (PBS), re-incubated for 30 min and washed twice again. After co-culture, the cells were harvested for analysis by flow cytometer (Epics Altra HyperSort; Beckman Coulter, Fullerton, CA, USA) and confocal microscope (LSM 310 Pascal; Carl Zeiss, Oberkochen, Germany). The double-positive signals were considered to be BM-DC that phagocytized *S. schenckii*. In confocal microscope detection, only the cells after 60 min co-culture were used. For analysis using electron microscope, immature BM-DC were first co-cultured with *S. schenckii* for 60 min and then were fixed with 4% paraformaldehyde in PBS. After a serial of sample preparation steps including dehydration, embedding, sectioning and staining, the samples were then detected by electron microscope (Hitachi, Tokyo, Japan).

S. schenckii-pulsed BM-DC maturation

Immature BM-DC (1×10^6 cells) were co-cultured with yeast cells of either *S. schenckii* or *C. albicans* in the ratios of 1:1 or 1:10 at 37°C with 5% CO_2 for 24 h to induce fungus-pulsed BM-DC. The fungus-pulsed BM-DC were then collected and stained with R-PE-conjugated antimouse CD40 antibody (BD Biosciences), R-PE-conjugated antimouse CD80 antibody (US Biological, Swampscott, MA, USA), R-PE-conjugated antimouse CD86 antibody (Immunotech, Marseille, France) and FITC-conjugated antimouse CD11c antibody (BD Biosciences) for evaluation of the co-expression of CD11c with CD40, CD80 and CD86 by flow cytometer.

Cytokines secreted by *S. schenckii*-pulsed BM-DC

To evaluate the cytokine production by fungus-pulsed BM-DC, the immature BM-DC (1×10^6 cells) were co-cultured with yeast cells of either *S. schenckii* or *C. albicans* in the ratios of 2:1, 1:1, 1:2, 1:5 and 1:10. After 48 h co-culture, the supernatant was harvested for detection of IL-12p40, IL-12p70, interferon (IFN)-γ and IL-4 by enzyme-linked immunosorbent assays (ELISA), according to the manufacturer's instructions (R&D Systems).

T-cell proliferation induced by *S. schenckii*-pulsed BM-DC

To obtain *S. schenckii*-sensitized lymphocytes, *S. schenckii* lysate in Freund's complete adjuvant (Sigma-Aldrich, St Louis, MO, USA) was s.c. injected into the abdomen of BALB/c mice. Three months later, axillary and inguinal lymph nodes were dissected, minced and filtered to obtain cell suspensions. Effector lymphocytes were prepared from the cell suspensions using mouse T-cell enrichment columns (R&D Systems). BM-DC were co-cultured with *S. schenckii* in the ratio of 1:1 for 24 h to induce *S. schenckii*-pulsed BM-DC. Proliferation assay was done by co-culturing 5×10^4 *S. schenckii*-pulsed BM-DC with various ratios of effector lymphocytes for 48 h, and incorporation of (^{3}H)-thymidine for 18 h, as previously described.[10] Uptake of (^{3}H)-thymidine was measured by scintillation counter to reflect the proliferation ability of effector cells.

Statistical analysis

Statistical analysis was performed using Student's two-tailed *t*-test. A probability value less than 0.05 was considered to be statistically significant.

RESULTS

BM-DC phagocytosis of *S. schenckii*

To confirm the BM-DC phagocytosis of *S. schenckii*, the immature BM-DC and *S. schenckii* yeast cells were separately stained and co-cultured for 0, 15, 30 and 60 min. After co-culture, the BM-DC phagocytosis was first detected by flow cytometer. The result showed the double-positive cell numbers for FITC-MHC class II (BM-DC) and CMTPX (*S. schenckii*) increased with the co-culture time (Fig. 1a,b). Electron microscopy also detected the *S. schenckii* inside the BM-DC (Fig. 1c). To clearly exclude the possibility that the *S. schenckii* was just attached to the cell surface, confocal microscope analysis was employed to confirm the coexistence of the two signals (Fig. 1d). The cells were examined across their entire

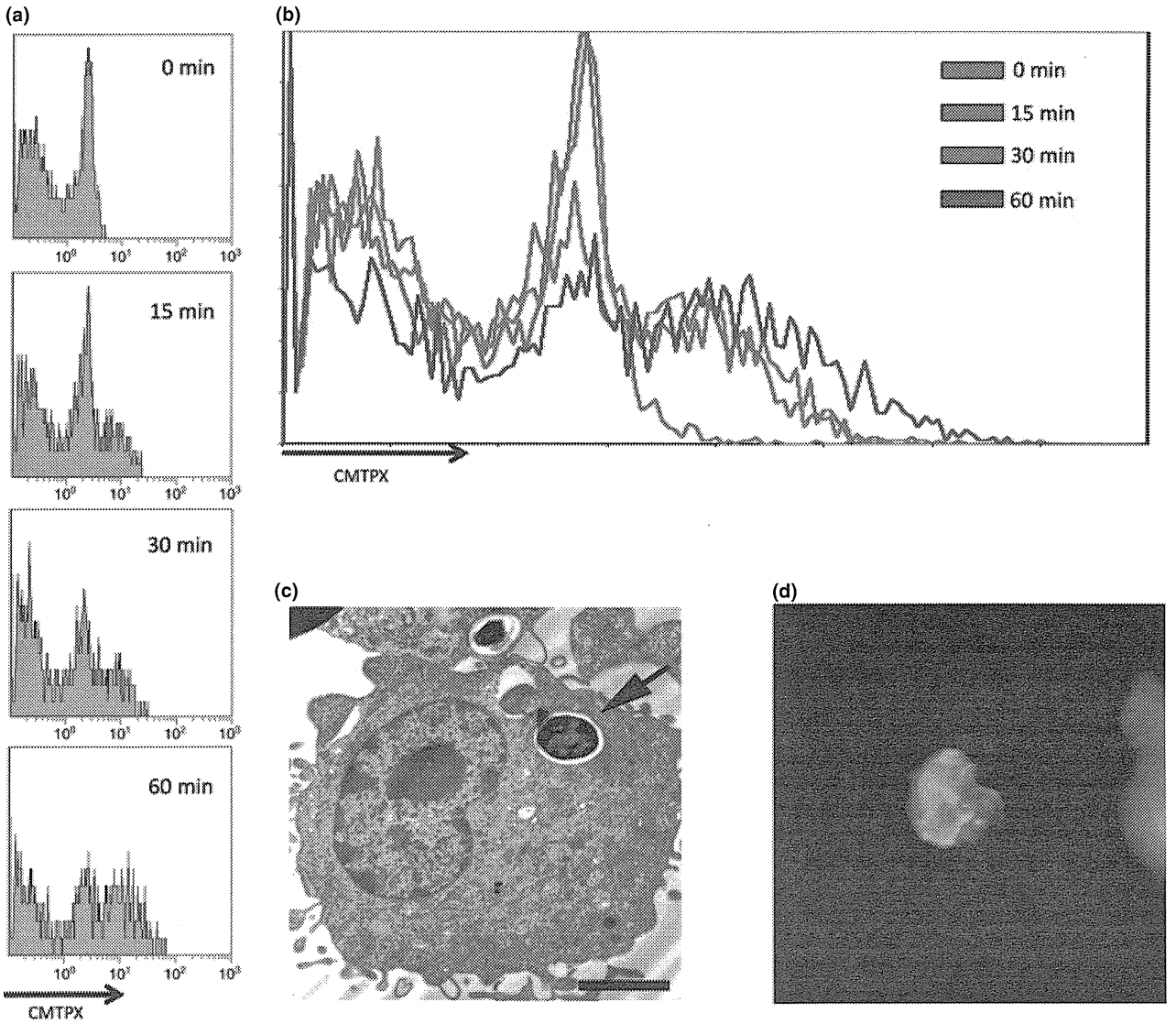

Figure 1. Bone marrow-derived dendritic cell (BM-DC) phagocytosis of *Sporothrix schenckii*. (a) BM-DC were detected by flow cytometer at indicated time points after co-culture with *S. schenckii*. After gating with fluorescein isothiocyanate (FITC) major histocompatibility complex (MHC) class II (BM-DC)-positive signals, the CMTPX (*S. schenckii*)-positive signals are shown. (b) Merged time course results. (c) BM-DC phagocytosis of *S. schenckii* detected by electron microscope. An arrow indicates the *S. schenckii* in BM-DC. Scale bar, 2 μm. (d) BM-DC phagocytosis of *S. schenckii* detected by confocal microscope. Red (CMTPX) and green (FITC) signals indicated *S. schenckii* and BM-DC, respectively. All experiments were performed in triplicate and representative results are shown.

diameter, which definitely demonstrated that the *S. schenckii* was inside the cells rather than just being attached to the cell surface.

BM-DC maturation stimulated by *S. schenckii*

To know the effect of *S. schenckii* on BM-DC maturation, immature BM-DC were co-cultured with either *S. schenckii* or *C. albicans* in the ratios of 1:1 or 1:10. After co-culture, the surface maturation markers, CD40, CD80 and CD86, were detected by flow cytometer. Compared to non-pulsed BM-DC, the increased expression of all three maturation markers was found in both *S. schenckii*-pulsed BM-DC and *C. albicans*-pulsed BM-DC (Fig. 2). Higher induction of surface maturation markers was detected in BM-DC co-cultured with *S. schenckii* or *C. albicans* in the ratio of 1:10 (Fig. 2).

Cytokines secreted by *S. schenckii*-pulsed BM-DC

To evaluate the cytokine production by fungus-pulsed BM-DC, the immature BM-DC (1×10^6 cells) were co-cultured with yeast cells of either *S. schenckii* or *C. albicans* in various ratios. The cultured supernatant was then detected by ELISA to analyze the IL-12p40, IL-12p70, IL-4 and IFN-γ levels.

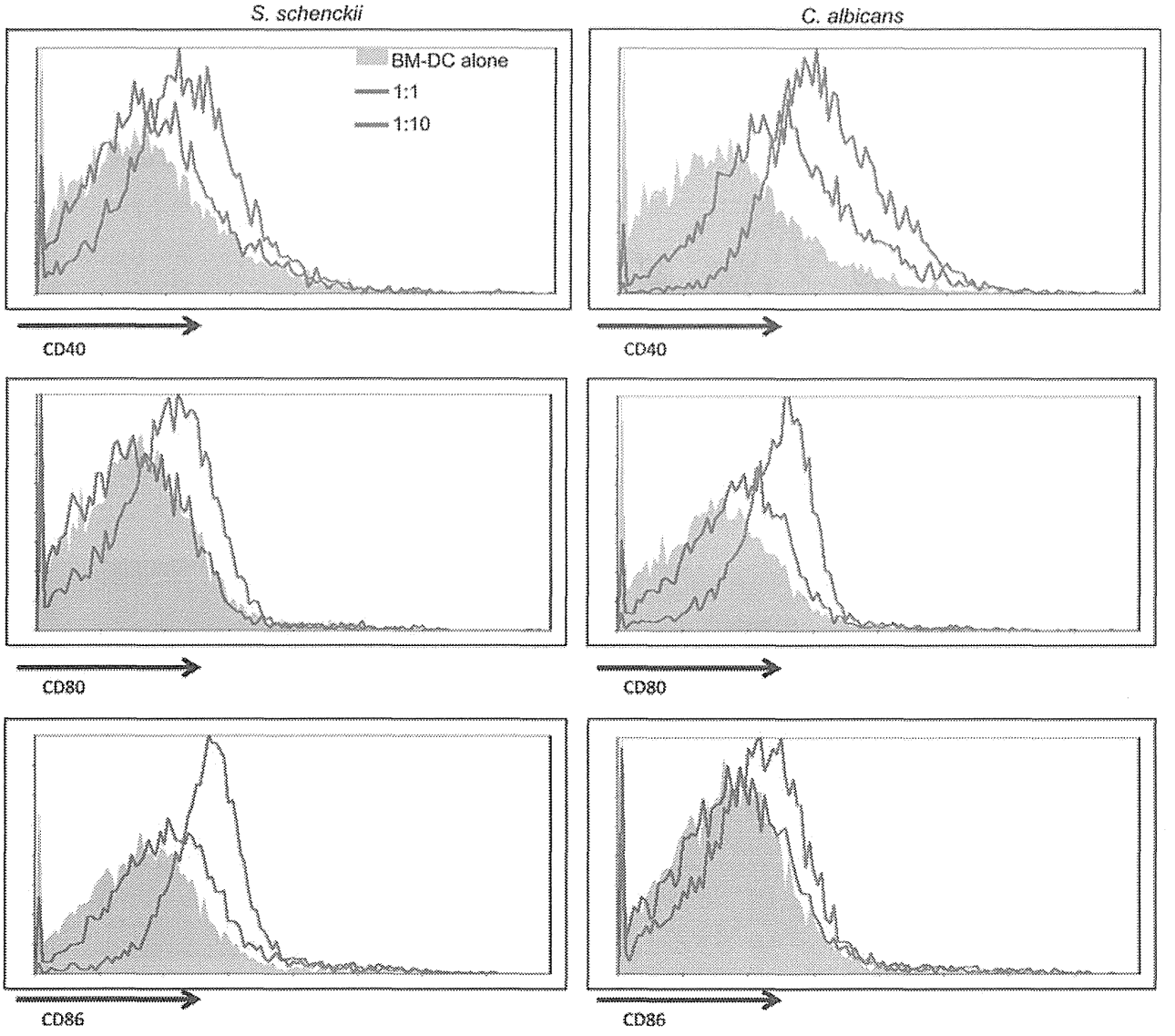

Figure 2. Expression of co-stimulatory molecules on *Sporothrix schenckii*-pulsed bone marrow-derived dendritic cells (BM-DC). Flow cytometer was employed to detect co-stimulatory molecule expression on the surface of fungus-pulsed BM-DC. After gating with fluorescein isothiocyanate (CD11c)-positive signals, the R-PE-positive signals for CD40 (upper panels), CD80 (middle panels) and CD86 (lower panels) molecules induced by *S. schenckii* (left) or *Candida albicans* (right) are shown. The experiment was done three times and representative results were shown in this figure.

Compared to non-pulsed BM-DC, *S. schenckii*-pulsed BM-DC produced significantly higher levels of IL-12p40 and the IL-12p40 level increased with the number of fungi used (Fig. 3a). However, IL-12p40 production in *S. schenckii*-pulsed BM-DC was much lower than that in *C. albicans*-pulsed BM-DC (Fig. 3a). Compared to non-pulsed BM-DC, *S. schenckii*-pulsed BM-DC also produced slightly higher levels of IL-12p70 in a fungus dose-dependent manner, although the difference cannot reach the statistical significance (Fig. 3b). In contrast, *C. albicans*-pulsed BM-DC produced significantly higher levels of IL-12p70, compared to non-pulsed BM-DC (Fig. 3b). The IL-4 and IFN-γ expression was not detectable in the supernatant of both *S. schenckii*-pulsed BM-DC and non-pulsed BM-DC (data not shown). Compared to *S. schenckii*, *C. albicans* induced more vigorous cytokine production by BM-DC.

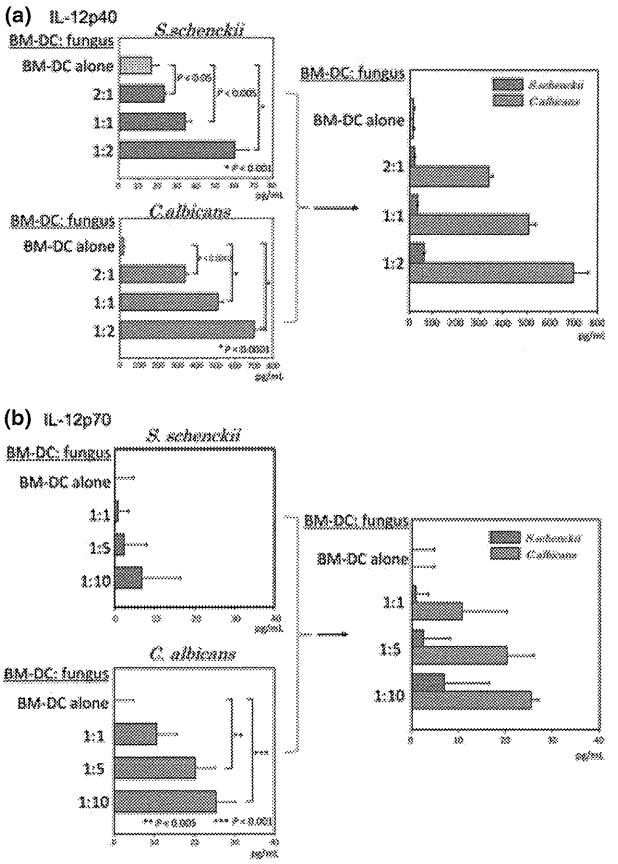

Figure 3. Cytokines produced by *Sporothrix schenckii*-pulsed bone marrow-derived dendritic cell (BM-DC). (a) Interleukin (IL)-12p40 enzyme-linked immunoassay (ELISA) results for *S. schenckii*-pulsed BM-DC (upper left panel) and *Candida albicans*-pulsed BM-DC (lower left panel). Different scales are used in these two panels. In the right panel, results for both *S. schenckii* and *C. albicans*-pulsed BM-DC are shown together in the same scale to compare the results directly. (b) IL-12p70 ELISA results for *S. schenckii*-pulsed BM-DC (upper left panel) and *C. albicans*-pulsed BM-DC (lower left panel). In the right panel, results for both *S. schenckii* and *C. albicans*-pulsed BM-DC are shown together. Each experiment was done in triplicate. The average results of three independent experiments were used as the final results (mean ± standard deviation).

T-cell proliferation induced by *S. schenckii*-pulsed BM-DC

To investigate the antigen-presenting ability of BM-DC, we measured the uptake of (^{3}H)-thymidine in mixed leukocyte reaction using *S. schenckii*-pulsed BM-DC and effector lymphocytes from mice pre-sensitized with *S. schenckii*. As shown in Figure 4, *S. schenckii*-pulsed BM-DC induced significantly higher levels of proliferation of effector lymphocytes, compared to non-pulsed BM-DC. The proliferation activities increased with the number of *S. schenckii* used.

DISCUSSION

Dendritic cells are derived from bone marrow progenitors and circulate in the blood as immature precursors. Upon appropriate stimulation, like fungus infection, DC undergo further maturation and migrate to secondary lymphoid tissues, where they present antigens to T cells and induce an immune response. The type of immune response initiated by DC ultimately depends on the type of maturation signal, pathogens and environment encountered.[6] In the present study, we proved that BM-DC are able to take up the antigen by phagocytosis of *S. schenckii*, undergo maturation program shown by expressing co-stimulator factors (CD40, CD80 and CD86), and induced the immune responses by secreting cytokines like IL-12 and stimulating the proliferation of pre-sensitized T lymphocytes.

Sporothrix schenckii is a thermo-dependent dimorphic fungus, which has two forms. The hyphal form mainly existing in the normal environment can enter the body through either traumatic implantation or inhalation.[11,12] Another is the yeast form

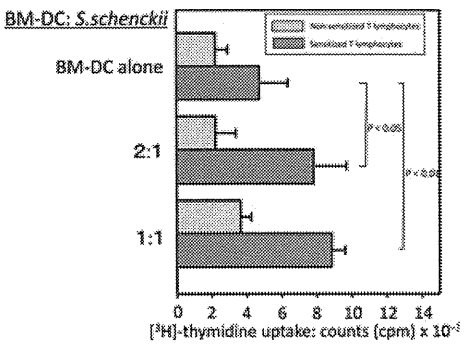

Figure 4. T-cell proliferation induced by *Sporothrix schenckii*-pulsed bone marrow-derived dendritic cells (BM-DC). *S. schenckii*-pulsed BM-DC were co-cultured with *S. schenckii*-sensitized lymphocytes in the ratio of 2:1 and 1:1 for 48 h. (^{3}H)-thymidine was then added into the co-culture system for another 18 h culture. The uptake of (^{3}H)-thymidine was used to evaluate the effector T-cell proliferation ability. Each experiment was done in triplicate. The average results of three independent experiments were used as the final results (mean ± standard deviation).

developed at 37°C. In biopsies or excised specimens, *S. schenckii* is observed only as the yeast form. This is why we selected the yeast form of *S. schenckii* in our experiments. In the present study, we first proved that BM-DC can phagocytize the *S. schenckii* by three different methods. These results also suggested that phagocytosis of *S. schenckii* may contribute to the maturation induction. However, the possibility that other pathways are involved in the BM-DC maturation program cannot be excluded.[7]

Dendritic cells are potent stimulators of the immune response. Most importantly, they are capable of activating naive T cells by expressing high levels of co-stimulatory molecules, like CD40,CD80 and CD86.[13] The expression of co-stimulatory molecules on cell surface is also used as a marker for DC maturation.[13,14] To analyze maturation stages of *S. schenckii*-pulsed BM-DC, a flow cytometer was employed to examine the expression of CD40, CD80 and CD86 on the *S. schenckii*-pulsed BM-DC. The increased expression of co-stimulatory molecules (CD40, CD80 and CD86) on the cell surfaces of BM-DC indicated that BM-DC became mature in the presence of *S. schenckii* antigens.[15]

To know the tendency of immune responses induced by *S. schenckii*-pulsed BM-DC, ELISA was used to detect the cytokines produced in the supernatant of *S. schenckii*-pulsed BM-DC. Higher production levels of IL-12p40 and IL-12p70, but not IL-4 and IFN-γ, were found. IL-12 and IFN-γ preferentially induce differentiation of naive T cells to Th1 cells.[16,17] Inversely, IL-4 is representative of a T-helper (Th)2 response.[18] Therefore, our results suggested that *S. schenckii*-pulsed BM-DC induced Th1 prone immune responses. This result is consistent with previous reports, although there are some differences in experimental details, such as the *S. schenckii* strain used and the mouse species for BM-DC isolation.[8,19]

Clinically, *S. schenckii* causes cutaneous sporotrichosis in immunocompetent hosts, in which the spores of *S. schenckii* can survive and proliferate in the dermis of the host. In contrast, *C. albicans* cannot cause deep candidiasis in immunocompetent hosts. The difference may reflect the response of dendritic cells to the two fungi. Compared to *S. schenckii*, *C. albicans* induced more vigorous cytokine production by BM-DC. This result indicated that *S. schenckii* can induce the BM-DC maturation to a certain extent, but not so completely as *C. albicans*. This may help to explain the differences between their clinical manifestations.

Finally, this study revealed that *S. schenckii*-pulsed BM-DC induced proliferation of lymphocytes, which were previously sensitized by *S. schenckii* lysate. To our knowledge, this is the first report that BM-DC can activate the *S. schenckii*- sensitized T lymphocytes. This result implied that dermal DC-phagocytized *S. schenckii* may modulate inflammation through activation of antigen-sensitized lymphocytes in cutaneous *S. schenckii* infection, particularly in individuals who were previously sensitized with this fungus.

In *Sporotrichosis*, the major clinical manifestations occur in the skin. However, cases with visceral lesions also have been increasingly reported particularly in immune-compromised patients. The different virulence of individual *S. schenckii* strains has also been suggested to contribute to form such different clinical manifestations.[8] Therefore, it is interesting to know whether dermal DC in the participates in the same manner in immune responses induced by *S. schenckii* strains of different virulence.

Until now, there have been only two studies researching the mechanism of how DC are involved in the immune responses against *S. schenckii*.[8,19] One used mouse BM-DC similar to ours, and another used human monocyte-derived DC. All three studies including our present one conducted experiments *in vitro*. Therefore, it should also be clarified in future whether BM-DC and human monocyte-derived DC *in vitro* are suitable substitutes of dermal DC *in vivo*.

In summary, we proved that BM-DC are able to phagocytize the *S. schenckii*, undergo the mature program, secret cytokines like IL-12, and further stimulate the proliferation of sensitized lymphocytes *in vitro*. All the results implied that dermal DC may participate in the induction of immune responses against *S. schenckii* infection in sporotrichosis.

ACKNOWLEDGMENTS: We gratefully appreciate the secretarial work of Ms Tomoko Tashima, Ms Mihoko Ikeda, Ms Mami Nishida and Ms Shoko Nakamura. We thank the patients for their participation. This study was supported by Grants-in-Aid for Scientific Research (nos. 20390308, 20591331, 21659271, 23591634, 23791298, 23791299, 23791300, 23791301, 24659534, 24591672, 24591640 and 24791185), and the Supported Program for the Strategic Research Foundation at Private Universities from the Ministry of Education, Culture, Sports, Science and Technology; and by "Research on Measures for Intractable Diseases" Project: matching fund subsidy (H23-028 to K. I., and H24-038 to T. H.) from the Ministry of Health, Labor and Welfare. The study was also supported by grants from the Kaibara Morikazu Medical Science Promotion Foundation, Ishibashi Foundation, Kanae Foundation for the Promotion of Medical Science, Takeda Science Foundation, Chuo Mitsui Trust and Banking Company and Nakatomi Foundation.

CONFLICT OF INTEREST: The authors declare no conflicting interests.

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DOI: 10.1111/exd.12317
www.wileyonlinelibrary.com/journal/EXD

Signalling pathways in pemphigus vulgaris

Xiaoguang Li, Norito Ishii, Chika Ohata, Minao Furumura and Takashi Hashimoto

Department of Dermatology, Kurume University School of Medicine and Kurume University Institute of Cutaneous Cell Biology, Kurume, Fukuoka, Japan

Correspondence: Takashi Hashimoto, MD, Department of Dermatology, Kurume University School of Medicine, and Kurume University Institute of Cutaneous Cell Biology, 67 Asahimachi, Kurume, Fukuoka 830-0011, Japan, Tel.: +81 942 31 7571, Fax: +81 942 34 2620, e-mail: hashimot@med.kurume-u.ac.jp

Commentary for article entitled 'EGFR inhibiters erlotinib and lapatinib ameliorate epidermal blistering in pemphigus vulgaris in a non-linear, V-shaped relationship', by Sayar BS, Ruegg S, Schmidt E, Sibilia M, Siffert M, Suter MM, et al., which was recently published in Exp Dermatol.

Abstract: Acantholysis in pemphigus vulgaris is induced by binding of autoantibodies to desmoglein 3 (Dsg3). The roles of signalling pathways on development of acantholysis have recently been extensively studied. In the study by Sayar *et al.*, recently published in Exp Dermatol, epidermal growth factor receptor (EGFR) signalling was activated in both *in vivo* and *in vitro* pemphigus vulgaris experimental models. However, while EGFR inhibitors suppressed activity of p38 mitogen-activated protein kinase (p38MAPK) linearly, they suppressed activity of c-Myc and acantholysis in a non-linear, V-shaped relationship. These findings indicated complicated interactions among EGFR, p38MAPK and c-Myc in pemphigus vulgaris pathology.

Key words: acantholysis – pemphigus vulgaris – signalling pathway

Accepted for publication 30 December 2013

Pemphigus vulgaris (PV) is an autoimmune bullous disease characterized by acantholysis caused by autoantibodies against desmosomal cadherin, desmoglein 3 (Dsg3) (1,2). Desmosomal cadherins mediate intercellular adhesion by either haemophilic binding or heterophilic binding between Dsgs and desmocollins (3).

Because the antigen itself is cell adhesion molecule, steric hindrance by autoantibody binding to Dsg3 was first considered as the major mechanism for acantholysis (4). However, accumulating evidences suggested that the autoantibody-induced acantholysis in PV involves more complicated processes. Autoantibody binding to Dsg 3 on the cell surface may result in Dsg3 endocytosis, which weakens intercellular adhesion and finally leads to acantholysis in the epidermis (3).

Thereafter, a number of studies suggested the involvement of multiple signalling events in the pathogenesis of PV(3,5,6). Most of these studies used two experimental disease models: that is, *in vivo* passive transfer neonatal mouse model and *in vitro* dissociation assay using normal human, normal mouse or HaCaT keratinocytes, which was induced by the addition of either PV-IgG or AK23, a mouse pathogenic anti-Dsg3 monoclonal antibody (4,7–9).

Although a large number of studies addressed the importance of signalling pathways on the pathogenesis of PV, the current evidences are still not enough to fully understand the mechanism and contribution of signalling pathways in the development of PV acantholysis. In addition, the results in previous studies were inconsistent in different types of inhibitors, different doses of the inhibitors and different *in vivo* and *in vitro* experimental models of PV.

Previous studies examined effects of p38 mitogen-activated protein kinase (p38MAPK), c-Myc and epidermal growth factor receptor (EGFR), as well as plakoglobin and protein kinase C, and suggested abnormal activation or inhibition of these signalling pathways may contribute to acantholysis and blister formation in PV (Fig. 1a).

Among these signal pathways, p38MAPK signalling has been most extensively studied. p38MAPK signalling was activated in both PV mouse model and PV patient skin (10–12). Addition of inhibitors of p38MAPK prevented blister formation in mouse model (13,14). However, unexpectedly, addition of pathogenic antibodies still induced blister in mice lacking major p38MAPK isoform (15).

c-Myc, which is known to induce stem cells proliferation and terminal differentiation in keratinocytes, is another target molecule in pathogenesis of PV (16). c-Myc expression increased in both PV mouse model and PV patients (8). In addition, c-Myc inhibitors prevented acantholysis in both mouse model and dissociation assay (8).

EGFR signalling pathway was also activated in both mouse model and dissociation assay, and EGFR inhibitors prevented blister formation in mouse model, but not cell dissociation in cultured keratinocytes (17,18).

In addition, plakoglobin was found to be phosphorylated and translocated after binding of pathogenic antibodies to Dsg3, leading to loss of adhesion in cultured cells (8,19,20). Furthermore, the possible role of protein kinase C in blister formation in PV was also reported (21,22).

In the study by Sayar *et al.* (23), recently published in Exp Dermatol, two EGFR inhibitors, erlotinib and lapatinib, with different dosages were examined for their suppressive effects in both mouse model and dissociation assay of PV. Administration of both EGFR inhibitors at median doses, which reduced EGFR activity to 36–69% of normal level, suppressed blister formation. Low doses of both inhibitors did not show the suppressive effects. However, curiously, administration of both inhibitors at high doses, which reduced significantly EGFR activity, could not suppress acantholysis in mouse model (Fig. 1b). Similar results were also found in *in vitro* assay of PV.

Precisely, administration of low doses of the two EGFR inhi bitors decreased only slightly the activity of both p38MAPK and

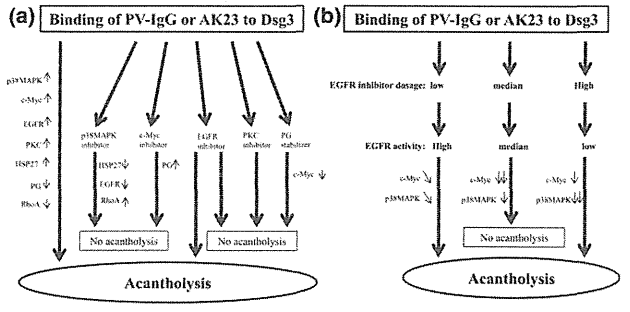

Figure 1. Diagram of main signalling pathways in PV acantholysis. (a) Summary of the results in previous studies of the role of main signalling pathways, including p38MAPK, c-Myc, EGFR, protein kinase C (PKC) and plakoglobin (PG). Binding of antibody (PV-IgG or AK23) to Dsg3 resulted in acantholysis in both *in vivo* and *in vitro* experimental PV models. Inhibitors specific to individual signalling pathways could prevent the acantholysis. (b) Summary of the results in the study of effects of EGFR inhibitors by Sayar *et al.* EGFR activity induced acantholytic change in a non-linear, V-shaped relationship. EGFR regulates differently the activities of p38MAPK signalling and c-Myc signalling.

c-Myc. Median doses of the EGFR inhibitors decreased significantly the activities of both p38MAPK and c-Myc. Unexpectedly, high doses of the inhibitors reduced significantly p38MAPK activity, but c-Myc activity was higher than the level of c-Myc in mice given with median doses of EGFR inhibitors (Fig. 1b).

Thus, although high doses of EGFR inhibitors could decrease activities of both EGFR and p38MAPK, acantholysis still occurred in the condition. This result implied that blister formation induced at low EGFR activities may develop through non-p38MAPK pathway. Conversely, after administration of EGFR inhibitors, the decrease in c-Myc activity paralleled well to suppression of acantholysis, indicating more important role of c-Myc on development of PV acantholysis than p38MAPK.

The non-linear, V-shaped effect of EGFR inhibitors indicated that median dose, but not higher or lower dose, of EGFR inhibitors may be beneficial for the treatment of pemphigus vulgaris. The mechanism of this curious effect is currently unknown. However, dermatologists should use inhibitors of EGFR or other signalling pathways more cautiously in the future therapy of PV, as both side effects and therapeutic effect may show a non-linear relationship to the dosages.

In addition, the results of the study by Sayar *et al.* may well explain the two contradictory results mentioned above. Thus, the discrepant result that mice lacking major p38MAPK isoform still showed acantholysis (15) may be explained by the possibility suggested by the study by Sayar *et al.* that EGFR may activate non-p38MAPK signalling, which induces PV acantholysis. Therefore, the effects of p38MAPK inhibitors in different doses should be re-examined in various PV models. In addition, the results of the study by Sayar *et al.* may also explain the inconsistent results that EGFR inhibitors prevented blister formation in mouse model but not in dissociation assay (17,18).

EGFR was previously suggested as one of downstream kinases of p38MAPK, because p38MAPK inhibitor inhibited EGFR activity (24,25). Sayar *et al.* (23) showed that EGFR inhibitor, erlotinib, inhibited the p38MAPK activity in parallel to activity of EGFR signalling. This result also indicated the presence of the cross-talk between p38MAPK and EGFR pathways. However, future studies are needed to clarify the interaction of these two signalling pathways, as well as most relevant pathogenic signalling pathways in PV acantholysis.

Acknowledgements

Writing of this commentary was supported by Grants-in-Aid for Scientific Research and Supported Program for the Strategic Research Foundation at Private Universities 2011–2015 from the Ministry of Education, Culture, Sports, Science and Technology.

Author contributions

Takashi Hashimoto and Xiaoguang Li prepared the manuscript. Norito Ishii, Chika Ohata and Minao Furumura critically revised the manuscript.

Conflict of interests

The authors declare no conflicting interests.

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DOI: 10.1111/exd.12447
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Human dermal fibroblast migration induced by fibronectin in autocrine and paracrine manners

Xiaoguang Li[1], Hua Qian[1], Fumitake Ono[1], Atsunari Tsuchisaka[1], Rafal P. Krol[1], Koji Ohara[1], Taihei Hayakawa[1], Satoko Matsueda[2], Tetsuro Sasada[2], Chika Ohata[1], Minao Furumura[1], Takahiro Hamada[1] and Takashi Hashimoto[1]

[1]Department of Dermatology, Kurume University School of Medicine, and Kurume University Institute of Cutaneous Cell Biology, Kurume, Fukuoka, Japan; [2]Department of Immunology and Immunotherapy, Kurume University School of Medicine, Kurume, Fukuoka, Japan
Correspondence: Takashi Hashimoto, MD, Department of Dermatology, Kurume University School of Medicine, and Kurume University Institute of Cutaneous Cell Biology, 67 Asahimachi, Kurume, Fukuoka 830-0011, Japan, Tel.: +81-942-31-7571, Fax: +81-942-34-2620, e-mail: hashimot@med.kurume-u.ac.jp

Abstract: Although fibronectin (FN) is known as a chemoattractant for human dermal fibroblasts (HDFs), it is unclear whether HDF migration is stimulated by FN produced by HDFs (autocrine manner) or by keratinocytes (paracrine manner). In this study, we investigated HDF migration by Boyden chamber assay using conditioned media from HDFs and HaCaT cells (keratinocyte cell line). Immunoblotting and enzyme-linked immunosorbent assay revealed that FN existed in both conditioned media. Boyden chamber assay showed both conditioned media stimulated HDF migration, which was inhibited by anti-FN antibody. Antibodies to both integrin β1and β3 subunits inhibited HDF migration induced by HDF-conditioned medium almost completely and that by HaCaT cell-conditioned medium with 50–60%. These results suggested that HDF migration was stimulated by FN in both autocrine and paracrine manners. However, the mechanisms of HDF migration by FN, particularly the role of integrin β1 and β3 subunits, were slightly different between autocrine and paracrine manners.

Key words: *cell migration – fibronectin – human dermal fibroblasts – integrin*

Accepted for publication 12 May 2014

Background

Human dermal fibroblast (HDF) migration is regulated by binding of growth factors and extracellular matrices to cell surface receptors (1). Among the extracellular matrices, fibronectin (FN) was considered to be essential in HDF migration (1).

FN is a multifunctional glycoprotein present in both plasma and tissues and plays a key role in adhesion and migration in various cell types (2–5). FN is produced by many cell types, including HDFs (2). FN was first reported as a potent chemoattractant for HDFs in 1981 (6). Three distinct functional domains of FN, RGDS cell-binding, heparin-binding and alternatively spliced IIICS domains, are necessary and sufficient for HDF migration (7). FN is considered to bind to cell surface integrins and work in signal transduction from environment to cells (2,8). Particularly, FN was reported to stimulate HDF migration by binding of integrin β1 and β3 subunits on HDFs (9–15).

Questions addressed

The question was whether HDF migration is induced by FN in both autocrine and paracrine manners.

Experimental design

FN expression in HDF- and HaCaT cell-conditioned media was first confirmed by immunoblotting. HDF- and HaCaT cell-conditioned media were then used as sources of FN for autocrine and paracrine manners, respectively. Antibodies against FN were then employed to evaluate the HDF migration by FN in conditioned media using Boyden chamber assay. Then, expression of integrins on HDF was investigated. In addition, participation of integrin β1 and β3 subunits in HDF migration induced by conditioned medium was evaluated by inhibitory antibodies against integrin β1 and β3 subunits. All materials and methods were described in Data S1.

Results

Immunoblotting detected FN expression in both HDF- and HaCaT cell-conditioned media (Fig. 1a). Commercially available enzyme-linked immunosorbent assay determined FN concentrations of 265.1 and 35.7 ng/ml in HDF- and HaCaT cell-conditioned media, respectively. Therefore, both conditioned media were used to evaluate the FN function on HDF migration. HDF migration was first examined by purified human FN at different concentrations, which showed highest effect at 5 μg/ml (Fig. 1b).

We first determined optimized incubation time for HDF migration by 100% conditioned media from both HDF and HaCaT cells. HDF migration induced by HDF-conditioned medium reached 422 cells/10HPF and 421 cells/10HPF after 10 and 32 h of incubation, respectively (Fig. 1c). HDF migration induced by HaCaT cell-conditioned medium reached 45 cells/10HPF and 85 cells/10HPF after 10 and 24 h of incubation, respectively (Fig. 1d). Therefore, incubation times of 32 and 24 h were used in following experiments for HDF- and HaCaT cell-conditioned media, respectively. When HDF migration was stimulated using various concentrations of conditioned media, 100% concentration achieved the highest migration activity (Fig. 1e,f). Therefore, 100% concentration of both conditioned media was used in following experiments.

When anti-FN antibody was added to conditioned media, HDF migration induced by both conditioned media decreased with the

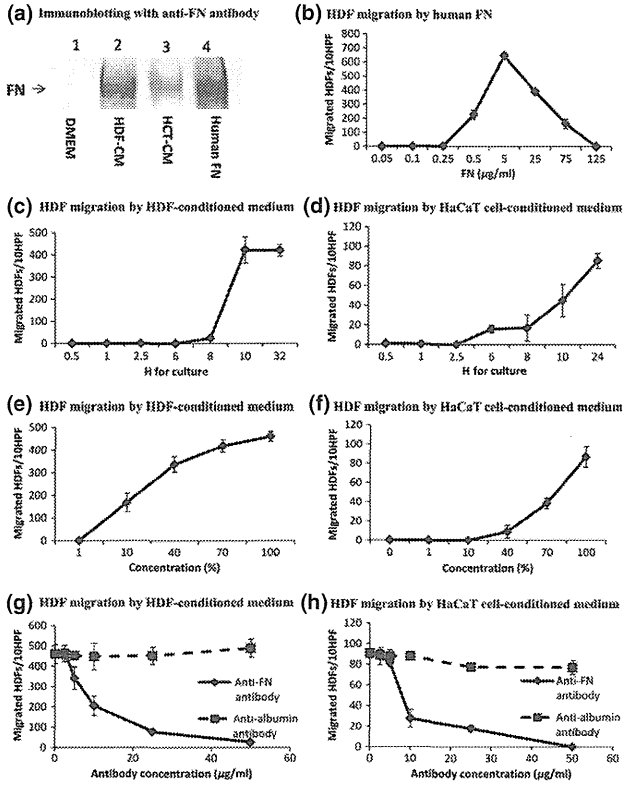

Figure 1. Results of immunoblotting and HDF migration studies. (a) Immunoblotting of conditioned media with anti-FN antibody. Lane 2: HDF-conditioned medium (HDF-CM). Lane 3: HaCaT cell-conditioned medium (HCT-CM). Human FN (lane 4) and DMEM (lane 1) were used as positive and negative controls, respectively. (b) HDF migration induced by various concentrations of purified human FN. (c,d) HDF migration by HDF-conditioned medium (c) and HaCaT cell-conditioned medium (d) at various incubation times. (e,f) HDF migration at various concentrations of HDF-conditioned medium (e) and HaCaT cell-conditioned medium (f). (g,h) Inhibitory effect of anti-FN antibody on HDF migration induced by HDF-conditioned medium (g) and HaCaT cell-conditioned medium (h). *Anti-albumin antibody was used as negative control, and the results* were shown by broken lines.

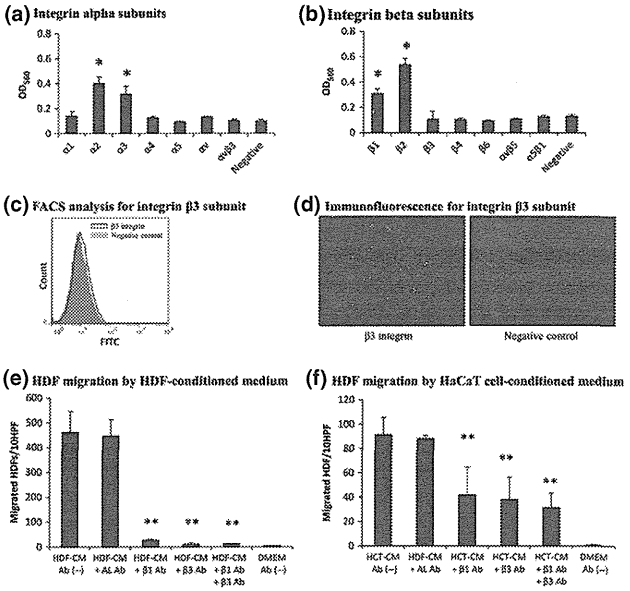

Figure 2. Expresson of various integrin subunits on HDFs and HDF migration inhibition by anti-integrin antibodies. (a,b) Expression of various integrin α and β subunits on HDFs detected by commercially available kit. Asterisks (*) indicate integrins with significantly higher expression ($P < 0.05$), when compared with the result in negative control wells bound by goat anti-mouse IgG. (c,d) Integrin $\beta3$ subunit on HDFs detected by FACS (c) and immunofluorescence (d). Samples stained with normal mouse IgG as the first antibody were used as negative control. (e,f) Inhibitory effect of antibodies to $\beta1$ or $\beta3$ subunit or combination of both antibodies on HDF migration induced by HDF-conditioned medium (e) and HaCaT cell-conditioned medium (f). HCT-CM: HaCaT cell-conditioned medium. HDF-CM: HDF-conditioned medium. $\beta1$ Ab: anti-integrin $\beta1$ antibody. $\beta3$ Ab: anti-integrin $\beta3$ antibody. Conditioned medium without antibodies was used as positive controls. DMEM without antibodies was used as negative control. Conditioned medium with anti-albumin antibody was used as IgG-negative control. AL Ab: anti-albumin antibody. Double asterisks (**) indicate significant difference ($P < 0.05$), when compared with positive controls (conditioned media without antibodies).

dose-dependent manner (Fig. 1g,h). Anti-FN antibody at 50 μg/ml inhibited HDF migration by HDF- and HaCaT cell-conditioned media by 94.3% and 99.9%, respectively (Table S1). These results indicated that FN was the main factor in both conditioned media for stimulation of HDF migration.

To investigate the participation of integrins into HDF migration by conditioned media, integrins on HDFs were detected by commercially available kit. HDFs strongly expressed integrin $\alpha2$, $\alpha3$, $\beta1$ and $\beta2$ subunits (Fig. 2a,b). In addition, additional FACS and immunofluorescence studies proved that integrin $\beta3$ subunit was also expressed relatively weakly on HDFs (Fig. 2c,d).

FN is known to induce HDF migration by binding to integrin $\beta1$ and $\beta3$ subunits (9–15). Therefore, we further examined the effect of integrin $\beta1$ and $\beta3$ subunits on HDF migration. HDFs suspension was plated in the upper chamber in the presence of antibodies to integrin $\beta1$ and $\beta3$ subunits or combination of both antibodies at concentrations ranging from 2 to 10 μg/ml.

Statistically significant inhibitory effect of antibodies to both $\beta1$ and $\beta3$ subunits and combination of both antibodies on HDF migration was detected at 10 μg/ml concentrations of antibodies ($P < 0.05$) (Fig. 2e,f and Table S1). Statistically significant difference in inhibitory effects of all anti-integrin antibody treatments on HDF migration was also found between HDF- and HaCaT cell-conditioned media ($P < 0.05$). In addition, anti-FN antibody treatment showed significantly higher inhibitory effects than those by anti-integrin antibody treatments in HaCaT cell-conditioned medium ($P < 0.05$) (Table S1).

Conclusions

This study showed that FN in HDF-conditioned medium (in autocrine manner) and HaCaT cell-conditioned medium (in paracrine manner) significantly stimulated HDF migration. This conclusion was further supported by results of additional studies that purified human FN stimulated HDF migration and that anti-FN antibody inhibited HDF migration induced by both conditioned media.

FN at dose of over 5 μg/ml displayed decreased activity on enhancement of HDF migration. This phenomenon was also reported in previous study (7). Although the real mechanism for this phenomenon is unknown, higher FN concentration may activate some signalling pathways, which inhibit HDF migration.

HDF migration induced by HaCaT cell-conditioned medium was less than that by HDF-conditioned medium. Different FN concentrations in both conditioned media and different migration assay incubation time might cause this result. Laminin-332 inhibited the cell migration induced by FN (16). In addition, HaCaT cell-conditioned medium was reported to contain laminin-332

(17). Therefore, laminin-332 and other contents in both conditioned media might also contribute to the different migration activity.

Both integrin $\beta 1$ and $\beta 3$ subunits may play essential role on HDF migration in autocrine manner, as antibodies to both integrin $\beta 1$ and $\beta 3$ subunits significantly inhibited HDF migration induced by HDF-conditioned medium. In addition, it was considered that even lower level integrin $\beta 3$ subunit on HDFs might have strong effects on FN-related HDF migration in cooperative manner with integrin $\beta 1$ subunit.

Antibodies to both integrin $\beta 1$ and $\beta 3$ subunits inhibited HDF migration induced by HaCaT cell-conditioned medium, although the effect was much less than that by HDF-conditioned medium. This result suggested that FN in HaCaT cell-conditioned medium might bind to other cell surface protein(s) on HDFs to induce HDF migration. Therefore, the mechanisms in FN-induced HDF migration in paracrine manner may be partially different from that in autocrine manner.

Acknowledgements

We gratefully appreciate the secretarial work of Ms. Tomoko Tashima and Ms. Mami Nishida. This study was supported by Grants-in-Aid for Scientific Research (No. 20390308, 20591331, 21659271, 23591634, 23791298, 23791299, 23791300, 23791301, 24659534, 24591672, 24591640, 24791185) and Supported Program for the Strategic Research Foundation at Private Universities from the Ministry of Education, Culture, Sports, Science and Technology and by "Research on Measures for Intractable Diseases" Project: matching fund subsidy (H23-028 to K. Iwatsuki, and H24-038 to T. Hashimoto) from the Ministry of Health, Labour and Welfare. The study was also supported by grants from the Kaibara Morikazu Medical Science Promotion Foundation, Ishibashi Foundation, Kanae Foundation for the Promotion of Medical Science, Takeda Science Foundation, Chuo Mitsui Trust and Banking Company, Limited and Nakatomi Foundation.

Author contributions

Xiaoguang Li, Hua Qian, Fumitake Ono, Atsunari Tsuchisaka, Rafal P. Krol, Koji Ohara, Taihei Hayakawa and Satoko Matsueda performed the experiments. Xiaoguang Li, Hua Qian, Atsunari Tsuchisaka and Takashi Hashimoto prepared the manuscript. Tetsuro Sasada, Chika Ohata and Minao Furumura contributed essential reagents and samples. Takahiro Hamada and Takashi Hashimoto originally designed this study. Takashi Hashimoto revised the manuscript.

Conflict of interests

The authors have declared no conflicting interests.

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Supporting Information

Additional supporting data may be found in the supplementary information of this article:

Data S1. Materials and methods.

Table S1. HDF migration inhibition rates by antibodies.

THE JOURNAL OF DERMATOLOGY

Late-onset Brooke–Spiegler syndrome with family histories of various cancers

Dear Editor,

Brooke–Spiegler syndrome (BSS) is an autosomal dominant hereditary disease, exhibiting multiple adnexal tumors, namely, cylindromas, trichoepitheliomas and spiradenomas. Mutation of the *CYLD* gene, a tumor suppressor located on chromosome 16, is responsible for BSS, and a number of mutations have been identified.[1] We report a late-onset case of sporadic BSS with family histories of various internal cancers.

A 57-year-old Japanese woman presented with a 6-year history of asymptomatic nodule on the scalp. The patient had no medical history, while hepatocellular carcinoma (HCC) developed in her father and sister, uterine cancer in her mother, gastric cancer in her brother and another sister, and renal cell carcinoma in another brother. Physical examination revealed a well-circumscribed nodule 10 mm × 13 mm in size (Fig. 1a), which was histopathologically confirmed as cylindroma (Fig. 1b). In the following 12 years, the patient developed cribriform trichoblastoma (trichoepithelioma) (Fig. 1c,d), cylindromas, spiradenoma (Fig. 1e,f) and cylindrospiradenoma (Fig. 1g,h) on various sites.

Because the patient described that her son had similar skin lesions, we performed sequence analysis of the *CYLD* gene using DNA samples taken from both peripheral blood and cylindrospiradenoma with the patient's informed consent and with approval of the ethics committee of Kurume University. Heterozygous and homozygous mutations (c.2272C>T, p.R758X) were

Correspondence: Takashi Hashimoto, M.D., Department of Dermatology, Kurume University School of Medicine, and Kurume University Institute of Cutaneous Cell Biology, 67 Asahimachi, Kurume, Fukuoka 830-0011, Japan. Email: hashimot@med.kurume-u.ac.jp

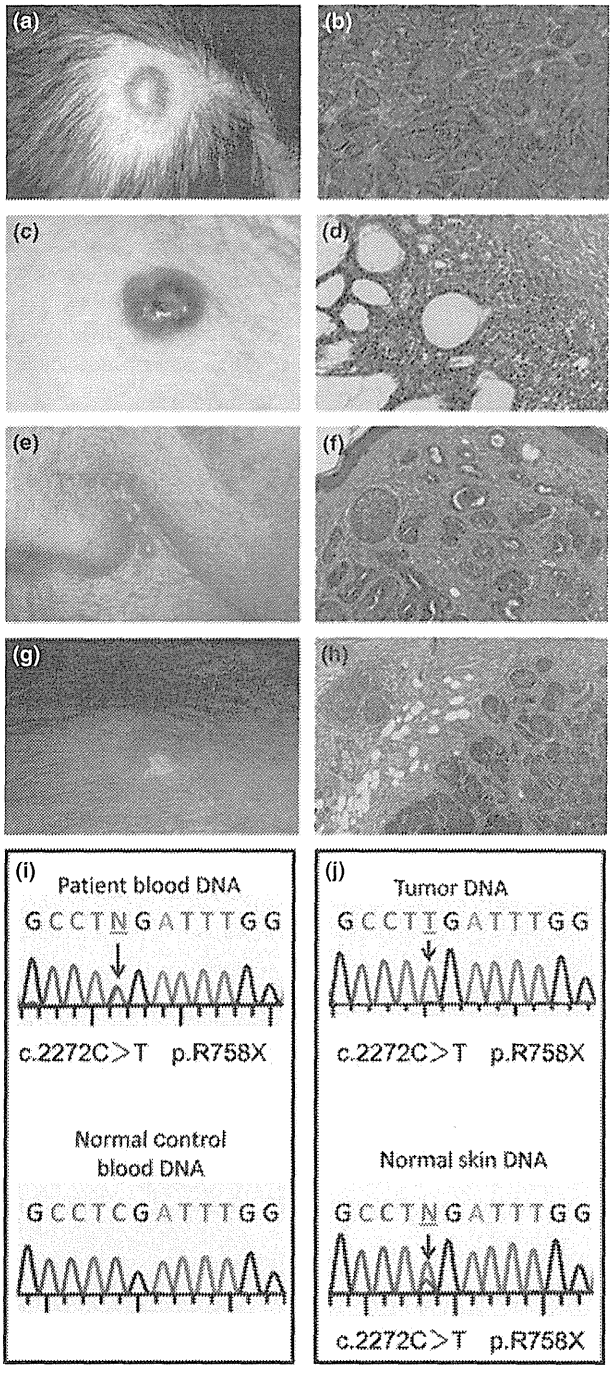

Figure 1. (a) Red nodule on the scalp at the first visit. (b) Histopathological examination of the skin lesion in (a) revealed relatively well-circumscribed discrete aggregations of basaloid cells, which were surrounded by rims of homogeneous basement membrane material and were situated in close proximity to one another with jigsaw puzzle appearance, in the dermis and subcutis (hematoxylin–eosin [HE], original magnification ×100). (c) Pigmented nodule on the cheek. (d) Histopathological examination of the skin lesion in (c) revealed large aggregations of basaloid cells with sieve-like pattern, surrounded by abundant fibrotic stroma with foci of follicular differentiation in the form of germs and rudimentary papillae at the periphery. The diagnosis of cribriform trichoblastoma (trichoepithelioma) was made (HE, ×200). (e) Small nodules next to ala nasi. (f) Histopathological examination of the skin lesion in (e) revealed aggregations composed of small dark cells and larger pale cells with lymphocytic infiltrate within the aggregations. The diagnosis of spiradenoma was made (HE, ×100). (g) Red nodule on the forehead at the third visit. (h) Histopathological examination of the skin lesion in (g) revealed areas typical of both cylindroma and spiradenoma. The diagnosis of cylindrospiradenoma was made (HE, ×100). (i) Mutation analysis of exon 17 of the *CYLD* gene from a peripheral blood sample of the patient identified a heterozygous mutation c.2272C>T. (j) The sequence analysis using tumor DNA showed only the mutant allele. The sequence analysis using DNA isolated from normal skin of the patient showed both mutant and wild-type alleles.

identified in exon 17 from peripheral blood DNA and tumor DNA, respectively (Fig. 1i,j). Loss of heterozygosity (LOH) in the tumor DNA was testified by microsatellite marker studies using D16S3136, D16S3034 and D16S3140, all of which locate around the *CYLD* gene locus. The patient refused genetic studies in other family members.

The present case is the second BSS case with LOH of 2272C>T in the *CYLD* gene. A recurrent nonsense mutation c.2272C>T has been identified in six BSS families.[1,2] Among these families, somatic mutations in tumor DNA were studied in nine tumors in three members from two families.[1] LOH of c.2272C>T was most commonly detected, while 2630delA was found in one trichoepithelioma. Intriguingly, the patient who had trichoepithelioma showing 2630delA also had another trichoepithelioma, which showed LOH of c.2272C>T. Similarly, each appendage tumor in our case might have had different somatic mutations.

CYLD is a deubiquitinating enzyme that downregulates the transcription factor nuclear factor (NF)-κB activated through specific tumor necrosis factor receptors.[3] Because NF-κB is required for appropriate cellular homeostasis of skin appendages, mutations in the *CYLD* gene are speculated to correlate with appendage tumor formation. NF-κB activation is also connected with multiple aspects of carcinogenesis, particularly in HCC and colon cancer.[4] Recently, CYLD mRNA expression was found to be reduced in HCC and colon cancer cell lines, and CYLD expression was reduced or lost in cell lines and tissues of HCC and colon cancer. These findings raised the possibility that the patient's family histories of various cancers may be related to loss of the *CYLD* gene function. Although malignant transformation of appendage tumors in BSS has occasionally been reported,[5] BSS cases with family histories of various internal cancers have never been reported. Further investigations are required to elucidate the role of NF-κB in BSS and various cancers.

ACKNOWLEDGMENTS: We gratefully appreciate the secretarial work of Ms Tomoko Tashima, Ms Mami Nishida and Ms Shoko Nakamura. We thank the patient for her participation.

CONFLICT OF INTEREST: None.

Mitsuhiro MATSUDA,[1,2] Chika OHATA,[1,2] Takahiro HAMADA,[1,2] Naoki OISO,[3] Daisuke TSURUTA,[4] Minao FURUMURA,[1,2] Takashi HASHIMOTO[1,2]

[1]*Department of Dermatology, Kurume University School of Medicine* [2]*Kurume University Institute of Cutaneous Cell Biology, Fukuoka,* [3]*Department of Dermatology, Kinki University Faculty of Medicine, Osaka-Sayama, and* [4]*Department of Dermatology, Osaka City University Graduate School of Medicine, Osaka, Japan*

doi: 10.1111/1346-8138.12451

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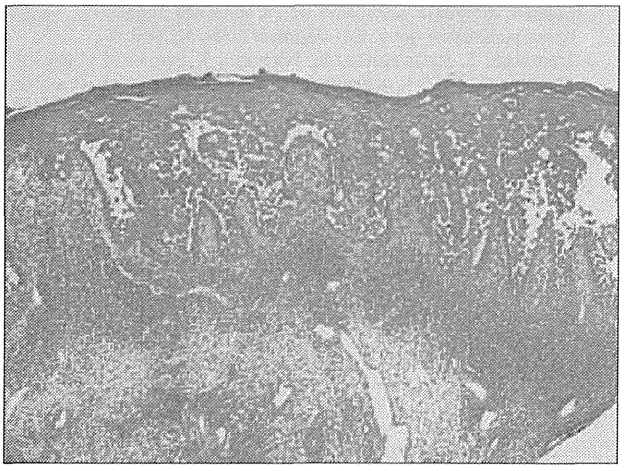

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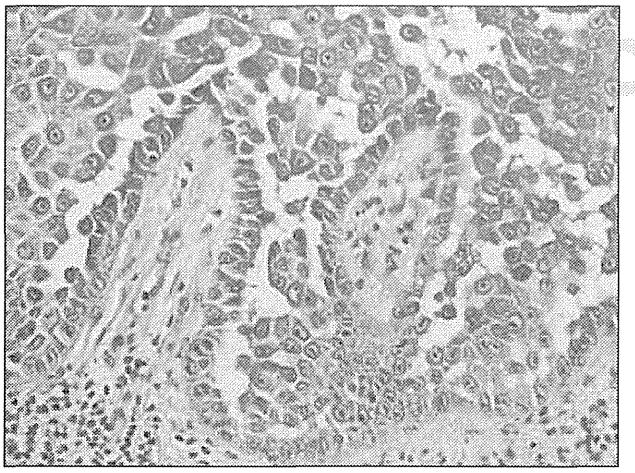

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The best diagnosis is:

a. Darier disease
b. Hailey-Hailey disease
c. herpesvirus infection
d. pemphigus foliaceus
e. pemphigus vulgaris

PLEASE TURN TO PAGE 33 FOR DERMATOPATHOLOGY DIAGNOSIS DISCUSSION

Chika Ohata, MD, PhD

From the Department of Dermatology, Kurume University School of Medicine, Japan.
The author reports no conflict of interest.
Correspondence: Chika Ohata, MD, PhD, Department of Dermatology, Kurume University School of Medicine, 67 Asahimachi, Kurume, Fukuoka, Japan 830-0011 (bboohay02@ybb.ne.jp).