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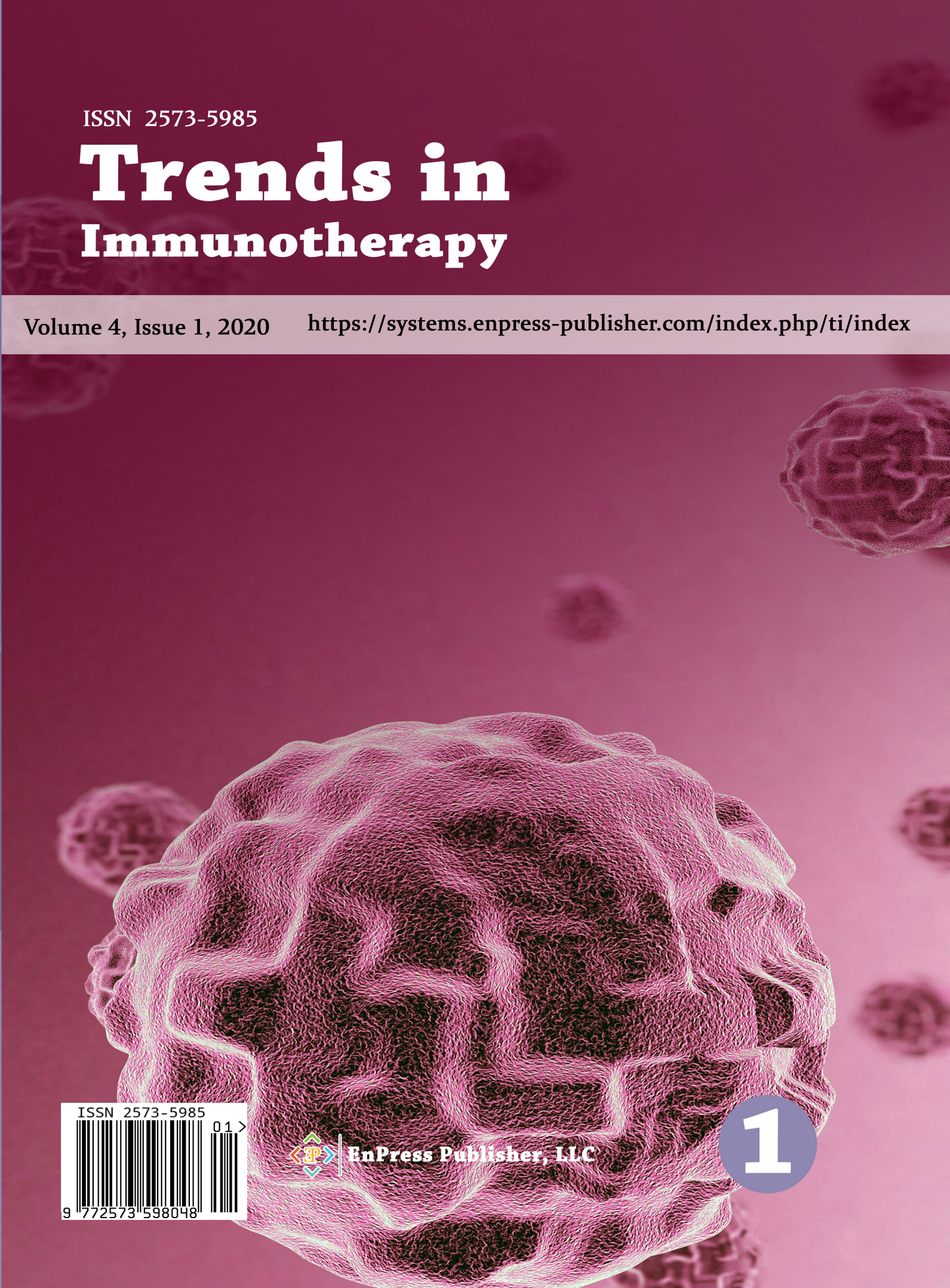

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Contents

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ORIGINAL ARTICLE

Combination use of triamcinolone acetonide and immunotherapy as a new therapeutic option in alopecia totalis

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ABSTRACT

Alopecia totalis (AT) with body hair loss is the most severe type of alopecia areata (AA). The ability to develop hair is suggested to be poor in such severe AA, because AT does not respond to corticosteroid pulses and immunotherapy using squaric acid dibutylester (SADBE) or diphenylcyclopropenone (DPCP). The purpose of this study is to assess the possibility of hair regrowth in AT with body hair loss. Ten patients with AT who did not respond to topical immunotherapies, received triamcinolone acetonide (TA) injections. Undiluted or 2-fold diluted solutions of TA were prepared and 0.1–0.2 mL of either of the two solutions was administered to each patient. In total, 2 mL of the selected solution was injected monthly into each area. In cases where vellus hair developed after the injections, we restarted the immunotherapy using SADBE or DPCP and continued the therapies for more than half a year. The development of vellus hair after TA injections was defined as a good response. Complete response rate to the topical injection of TA was 10% (1/10), however the partially good response rate was 60% (6/10). The good responders showed the anagen stage of hair follicle after TA injections. Furthermore, the complete responder to TA showed susceptibility to the subsequent immunotherapy and more regrowth of hair was seen. Even if patients with AT have suffered for a prolonged period since onset, it is possible to recover the hair cycle if they show susceptibility to intralesional corticosteroid and subsequent immunotherapy.

***Keywords**: alopecia totalis; triamcinolone acetonide; SADBE; DPCP; immunotherapy*

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Introduction

Alopecia areata (AA) is usually characterized by limited alopecic patches on the scalp. However, more severe forms may affect the entire scalp (alopecia totalis: AT) or body hair including eyebrows, cilia, axillary hair and hair of the pubis (alopecia universalis: AU). The possibility of hair regrowth is suggested to be poor in such AT with body hair loss, because AT does not respond to steroid pulses and immunotherapy using squaric acid dibutylester (SADBE) or diphenylcyclopropenone (DPCP)[1]. Intralesional corticosteroids are not as effective for the treatment of AT as they are for localized alopecia[2]. However, the recommended degree of intralesional corticosteroid is higher than other therapies in alopecia areata with up to 25 % hair loss[3]. It is supposed that the susceptibility of AT patients to intralesional corticosteroid is diverse. The purpose of this study is to assess the susceptibilities to intralesional corticosteroid and subsequent immunotherapy using SADBE or DPCP in AT patients with body hair loss.

Materials and methods

Ten patients with AT (male: 1, female: 9; aged 12–43 years) were enrolled in this study between 2008 and 2017. All of them had either partial or complete body hair loss. Topical immunotherapy using SADBE or DPCP for the AT patients was approved by the ethics committee of Wakayama Medical University. Informed consent was obtained from each patient. To exclude AA associated with other diseases, biological examinations were carried out including tests for serum zinc, serum iron, HBs antigen, and hepatitis C

antibody, a serologic test for *syphilis* and Treponema pallidum hemagglutination, and a thyroid function test and a measurement of anti nuclear antibody.

We started a modified topical immunotherapy using SADBE or DPCP starting with 0.01% for the patients with AT before starting triamcinolone acetonide (TA) injections[1]. The immunotherapy was continued at optimal concentration in each patient for several months. The optimal concentration was decided in each patient to had seen continuous erythema for 4 to 5 days after the topical immunotherapy. Undiluted or 2-fold diluted solution of TA was prepared and 0.1–0.2 mL of the either solution was injected into each area. The choice of solution was determined by the patient's reaction to the initial injection of a double-diluted solution. Those who showed no adverse side effects subsequently received the undiluted solution. In total, 2 mL of the solution was injected monthly at least several times into the AT lesion in each patient. Repeated TA injections at the same site were avoided to prevent skin atrophy. Susceptibility to TA was assessed by whether vellus hair had developed in the patients with AT. Irrespective of the development of vellus hair in the patients with AT after several TA injections, we restarted the immunotherapy using SADBE or DPCP and continued the therapy every two weeks for more than half a year. We assessed clinical changes over time. Skin biopsies were performed before or after the intralesional corticosteroid in some patients.

Results

In this study, the AT patients with body hair loss had an average duration of more than 5 years from the onset of alopecia (**Table 1**). No one showed susceptibility to the immunotherapy using SADBE or DPCP before starting TA injections. Complete response rate to the topical injections of TA was 10 % (1/10) (Figure 1a. b, Table 1) however the partially good response rate was 60% (6/10) (**Table 1**). The good responder showed the anagen stage of hair follicle after TA injection (**Figure 2A**) and trichoscopic examination showed the development of vellus hair without exclamation mark hair (**Figure 2B**). Furthermore, she showed susceptibility to the subsequent immunotherapy and more regrowth of hair was seen up to 10 months after starting TA injections (**Figure 1C–F**). Some of the AT patients were partially susceptible to TA injections (Figure 3a. b) and hair follicles in the anagen stage were seen (**Figure 3C**). Some of the partial responders to TA injections were also partially susceptible to the immunotherapy of SADBE or DPCP (**Table 1**), however such patients did not show complete hair regrowth satisfactorily. There were no obvious side effects to the discontinuation of the therapies using TA injections and the immunotherapy during the study.

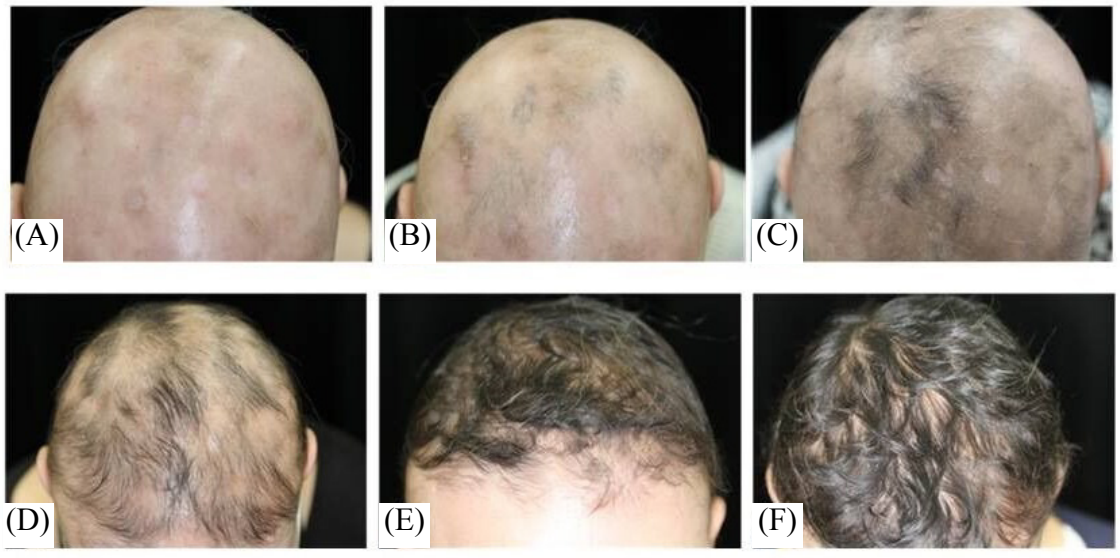

Figure 1. Clinical changes in alopecia totalis with body hair loss susceptible to intralesional triamcinolone acetonide (TA) and subsequent immunotherapy (Patient No. 1). (**A**) Alopecia totalis before starting intralesional TA injections. (**B**)Vellus hair was developed after monthly TA injections. (**C**) Immunotherapy using SADBE showed hair regrowth following TA injections. (**D-F**) Hair regrowth was accelerated by immunotherapy every two weeks from 6 months to 10 months after the TA injections.

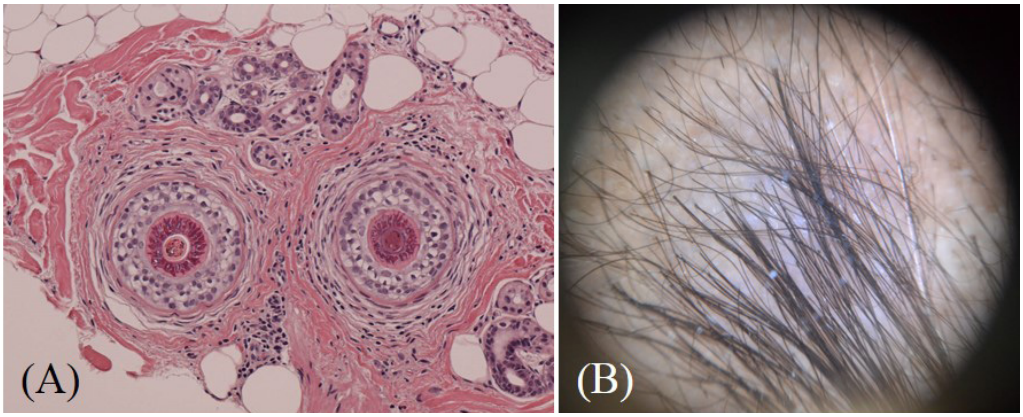

Figure 2. Histopathological finding and trichoscopic findings after the development of vellus hair. (Patient No. 1). (**A**) Hair follicles in the anagen stage were seen after two injections of half-diluted solution of TA (HE stain, ×200). (**B**) Trichoscopic finding showed the development of vellus hair without exclamation mark hair.

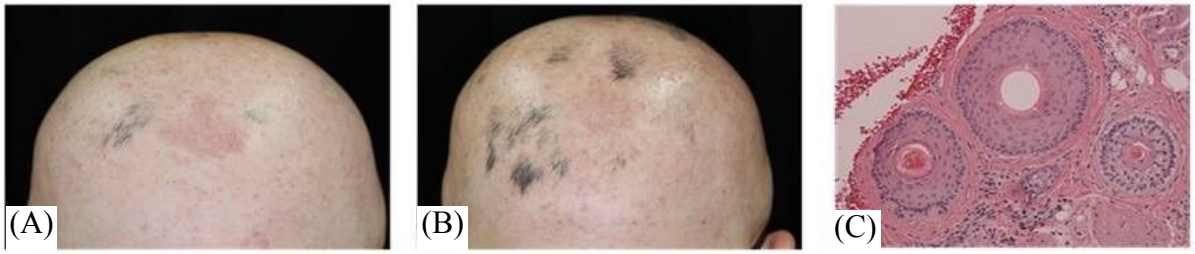

Figure 3. Clinical findings in AT with body hair loss partially susceptible to TA but not immunotherapy (Patient No. 7). (**A**) Alopecia totalis before starting an intralesional TA injection. (**B**) Intralesional injections of undiluted TA induced partial development of vellus hair. (**C**) Anagen hair follicles were seen after four times injections of undiluted solution of TA (HE stain, ×200).

Table 1. Summary of susceptibilities to both TA and immunotherapy in AT patients.

Pt No.	Age	Sex	Severity	Duration of alopecia (years)	Immuno-therapy	Susceptibility to immuno-therapy before TA injection	Susceptibility to TA injection (Vellus hair development)	Susceptibility to immuno-therapy after TA injection	Hair regrowth
1	41	F	S5B2	3	SADBE	-	+	+	+
2	28	F	S5B2	5	SADBE	-	+/-	+/-	+/-
3	18	F	S5B1	10	SADBE, DPCP	-	+/-	+/-	+/-
4	20	F	S5B2	3	DPCP	-	+/-	+/-	+/-
5	43	F	S5B1	3	DPCP	-	+/-	+/-	+/-
6	28	F	S5B1	4	DPCP	n.d.	+/-	-	-
7	37	M	S5B1	1	SADBE	-	+/-	-	-
8	42	F	S5B2	9	SADBE	-	-	-	-
9	26	F	S5B2	10	DPCP	-	-	-	-
10	12	F	S5B2	6	n.d.	n.d.	-	n.d.	-

(S5: 100% hair loss, B1: some body hair loss, B2: 100% body hair loss) (TA: triamcinolone acetonide) (Susceptibility; +: susceptible, +/-: partially susceptible, -; negative) (Hair regrowth; +: complete hair growth, +/-: partial hair growth, -: no hair growth)

Discussion

Various treatments are known in AT and alopecia universalis (AU), and although however certain treatments have shown significant hair regrowth, no treatment is completely effective[4]. Recently, the efficacy of hydroxychloroquine[5,6] in the treatment of AT has been reported[7] but its use for extensive alopecia areata is still controversial[8]. Tofatinib is also reported to show efficacy in severe AA including AT and AU[9].

In this study, the patients with AT had an average duration of more than 5 years from the onset of alopecia. Patients with AT for more than 6 months from the onset are resistant to steroid pulses and immunotherapy using SADBE or DPCP[1,10]. It is well known that the method using TA is most suitable for treating patchy hair loss of a limited extent. Despite this, because of the variability of individual response, we decided to try TA injections on the patients with AT. Susceptibility to TA is supposed to be diverse in AA including AT and AU, however intralesional corticosteroid is recommended in AA with up to 25 % hair loss[3]. One AT patient showed susceptibility to TA injections and complete vellus hair was seen in our study. Hair follicles in the anagen stage were seen histologically. The patient also showed susceptibility to the subsequent immunotherapy using SADBE, however she did not respond to it before the TA injections. Adequate vellus hair may be essential to in order to be susceptible to the subsequent immunotherapy. On the other hand, it is also possible that the susceptibility to immunotherapy sustains the anagen stage of the hair cycle. Even if patients with AT have suffered for a prolonged period since onset, it is possible to recover the hair cycle if they show susceptibility to intralesional corticosteroid and subsequent immunotherapy. The method using TA and subsequent immunotherapy for AT patients with body hair loss showed only a 10% success rate. However, as long as AT patients have a possibility to recover their hair, it is necessary to try various methods to recover the hair cycle even though the method has a low success rate.

Conflict of interest

This work was supported in part by a grant from Mitsubishi Tanabe Pharma to TY and a grant from Novartis Pharmaceuticals Corp. to NM.

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ORIGINAL ARTICLE

Anti-inflammatory effects of flavonoids in *Citrus jabara* fruit peels

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ABSTRACT

Currently, about half of people in Japan suffer from allergic diseases. Thus, *Citrus jabara* fruits have been paid attention as one of quite effective anti-allergic functional foods. *C. jabara* is an endemic species originally grown only in Kitayama village, Wakayama prefecture in Japan. Although genetic characterization and diversity of various *Citrus* fruits including *C. jabara* were researched, but there is room for the study on flavonoids characteristics in *C. jabara* fruit. For the alleviation of allergic symptom, anti-inflammatory effects are also important. In this study, characteristics of flavonoids in *C. jabara* fruit peels, and the anti-inflammatory effects of these purified flavonoids were investigated. Our results revealed that *C. jabara* is a unique *Citrus* that almost all of flavonoids in fruit peels was narirutin. There was no *Citrus* species with a flavanone glycosides content ratio like *C. jabara*. Although anti-inflammatory effects of narirutin was weak, but its aglycone naringenin exhibited following inhibitory effects: nitric oxide synthesis (IC_{50} = 105 μM), nitric oxide synthase induction, Interleukin-6 synthesis (IC_{50} = 65 μM), and inducible soluble epoxide hydrolase activity (IC_{50} = 267 μM). Since narirutin is deglycosylated to naringenin that is then absorbed by colonocytes, it is considered that narirutin exists like a prodrug and its aglycone naringenin works as an active form of anti-inflammatory effect in a living body at oral ingestion of *C. jabara* fruit peels.

Keywords: *Citrus jabara; anti-inflammatory effects; flavonoids; narirutin; naringenin*

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Introduction

Currently, about half of people in Japan suffer from allergic diseases such as pollen allergy, perennial rhinitis, and atopic dermatitis. So, it is figuratively said that allergic diseases are the current national disease in Japan. Under such condition, the functional foods to alleviate allergic symptoms, such as *Citrus unshiu* unripe fruit, were focused[1,2].

In recent years, *Citrus jabara* fruits has also been paid attention as one of quite effective anti-allergic functional foods. *C. jabara* is an endemic species originally grown only in Kitayama village, Wakayama prefecture in Japan, and is estimated as a natural hybrid species of *Citrus junos*, *Citrus reticulata*, and *Citrus kinokuni*[3]. *C. jabara* was recognized as a new variety of citrus by Dr. Tanaka in 1971, and then registered as a new nursery plant in 1979 by Mr. Fukuda (Japan the plant variety protection NO. 10). Concerning with anti-allergic activities of *C. jabara* fruits, the anti-degranulation activity[3], the improving effect of its juice on symptoms and quality of life in patients with Japanese cedar pollinosis[4], and the suppressive effect of its extracts on mucin production in human lung epithelial cells[5] were reported. Although genetic characterization and diversity of various citrus fruits including *C. jabara* were researched [6–8], but there is room for the study on flavonoids characteristics in *C. jabara* fruit. For the alleviation of allergic symptom, both anti-allergic effect and anti-inflammatory effects are important. Thus, in this study, we investigated the component analysis of flavonoids in *C. jabara* fruit peels and the anti-inflammatory effects of these isolated flavonoids.

Materials and Methods

Extract and isolation

C. jabara fruits were harvested in Kitayama village, Wakayama prefecture in Japan and on 3 November 2010. The residue of *C. jabara* fruits by commercial roller juice squeezer was used as *C. jabara* fruit peels. After drying, these peels were chopped and sealed in aluminum puck until use. Extract and isolation were carried out in 2010.

The dried of *C. jabara* fruit peels (1 Kg) were chopped into small pieces and extracted with *n*-hexane (3 L x 5), EtOAc (3 L x 3) and acetone (3 L x 3) using a ultrasonic extraction. The combined *n*-hexane, EtOAc and acetone extracts were concentrated to dryness in vacuo, respectively. The *n*-hexane residue (11.7 g) was subjected to column chromatography on silica gel and ODS, and eluted successively with a *n*-hexane – EtOAc and MeOH – H_2O solvent system to afford fourteen compounds, eight flavones: 3, 3', 4', 5, 6, 7, 8-heptamethoxyflavone (**FLV1**, 265.8 mg), natsudaidain (3-hydroxy-3', 4', 5, 6, 7, 8-hexamethoxyflavone) (**FLV2**, 130.3 mg), 5-hydroxy-3, 3', 4', 6, 7, 8-hexamethoxyflavone (**FLV3**, 29.3 mg), 3, 3', 4', 5, 6, 7-hexamethoxyflavone (**FLV4**, 14.6 mg), 8-hydroxy-3, 4', 5, 6, 7-pentamethoxyflavone (**FLV5**, 7.1 mg), 4', 5-dihydroxy-3, 3', 6, 7, 8-pentamethoxyflavone (**FLV6**, 2.9 mg), retusin (5-hydroxy-3, 3', 4', 7-tetramethoxyflavone) (**FLV7**, 1.9 mg), nobiletin (3', 4', 5, 6, 7, 8-hexamethoxyflavone) (**FLV8**, 1.9 mg), three flavanones: 3', 4', 5, 6, 7, 8-hexamethoxyflavanone (**FNN2**, 32.1 mg), 3', 4', 5, 6, 7-pentamethoxyflavanone (**FNN3**, 6.7 mg), 4', 5, 6, 7-tetramethoxyflavanone (**FNN4**, 1.4 mg), three chalcones: 2'-hydroxy-3, 3', 4, 4', 5', 6'-hexamethoxychalcone (**CHL1**, 31.7 mg), 2'-hydroxy-3, 4, 4', 5', 6'-pentamethoxychalcone (**CHL2**, 8.2 mg), 2'-hydroxy-4, 4', 5', 6'-tetramethoxychalcone (**CHL3**, 7.8 mg). The EtOAc and acetone residues were filtered to obtain an amorphous powder (narirutin: **FNN1**, 17.7 g). These compounds were determined by comparison of physical data and NMR spectral data with articles[9–13].

Chemical structures of isolated compounds were shown in **Figure 1**, and their names and amounts were listed in **Table 1**. Among these compounds, **FLV3, 5, 6, 7** and **CHL1-3** are the compounds which were isolated for the first time from *C. jabara* fruit peels[14]. Seven compounds that were obtained more than 10 mg (**FLV1 - 4**, **FNN1 - 2**, and **CHL1**) were evaluated in cell-based assays.

Materials

Naringenin was purchased from LKT Laboratory (St. Paul, MN, USA). Hesperidin, neohesperidin, and naringin were purchased from Nacalai Tesque

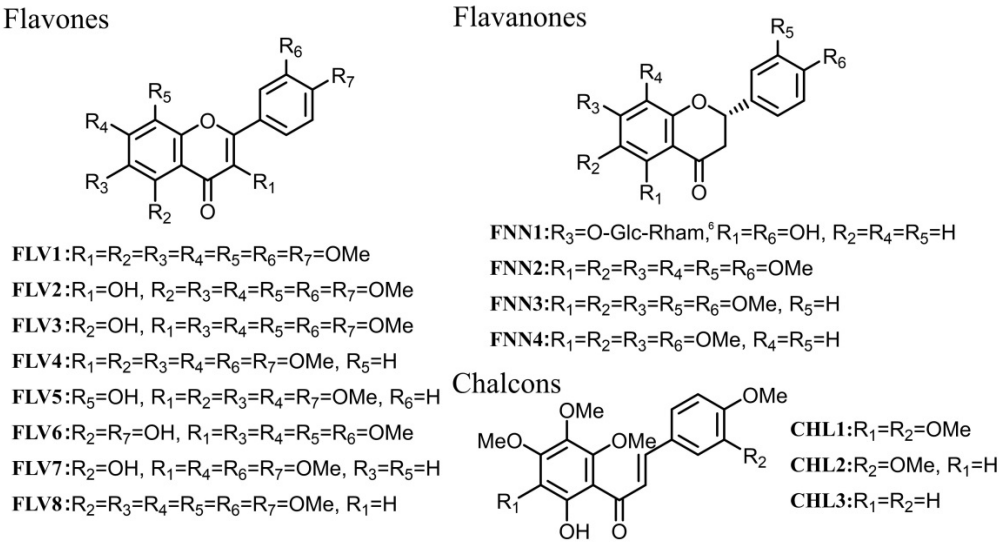

Figure 1. Structures of isolated flavonoids from dried *C. jabara* fruit peels

Table 1. Flavonoids isolated from dried *C. jabara* fruit peels (1 kg).

	Compound	Amount (mg)
Flavones		
FLV1	3, 3', 4', 5, 6, 7, 8-heptamethoxyflavone	265.8
FLV2	natsudaidain (3-hydroxy-3', 4', 5, 6, 7, 8-hexamethoxyflavone)	130.3
FLV3	5-hydroxy-3, 3', 4', 6, 7, 8-hexamethoxyflavone	29.3
FLV4	3, 3', 4', 5, 6, 7-hexamethoxyflavone	14.6
FLV5	8-hydroxy-3, 4', 5, 6, 7-pentamethoxyflavone	7.1
FLV6	4',5-dihydroxy-3, 3', 6, 7, 8-pentamethoxyflavone	2.9
FLV7	retusin (5-hydroxy-3, 3', 4', 7-tetramethoxyflavone)	1.9
FLV8	nobiletin (3', 4', 5, 6, 7, 8-hexamethoxyflavone)	1.9
Flavanones		
FNN1	narirutin	17,700.0
FNN2	3', 4', 5, 6, 7, 8-hexamethoxyflavanone	32.1
FNN3	3', 4', 5, 6, 7-pentamethoxyflavanone	6.7
FNN4	4', 5, 6, 7-tetramethoxyflavanone	1.4
Chalcones		
CHL1	2'-hydroxy-3, 3', 4, 4', 5', 6'-hexamethoxychalcone	31.7
CHL2	2'-hydroxy-3, 4, 4', 5', 6'-pentamethoxychalcone	8.2
CHL3	2'-hydroxy-4, 4', 5', 6'-tetramethoxychalcone	7.8

(Kyoto, Japan). Dulbecco's modified Eagle's medium (DMEM) and penicillin-streptomycin mixed solution (stabilized) were from Nacalai Tesque. Fetal bovine serum was obtained from Biowest (Nuaillé, France). Lipopolysaccharide (LPS) from *Escherichia coli* O55 was obtained from Wako Pure Chemical Industries (Osaka, Japan). Griess reagent system was obtained from Promega (Fitchburg, WI, USA). Cell Counting Kit-8 was from Dojindo Laboratories (Kumamoto, Japan). RIPA buffer and protease inhibitor cocktail were purchased from Wako Pure Chemical. BCA Protein Assay Kit was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Polyvinyliden fluoride (PVDF) membrane was obtained from Bio-Rad (Hercules, CA, USA). Blocking one, a blocking solution for blot membrane, was obtained from Nacalai Tesque. Rabbit antibody to inducible nitric oxide synthase (iNOS) was obtained from Sigma-Aldrich (St. Louis, MO, USA), and anti-rabbit IgG HRP-linked whole antibody was from GE Healthcare (Buckinghamshire, UK). Monoclonal antibody to β-actin HRP conjugate was obtained from Wako Pure Chemical Industries. Amersham ECL Prime Western Blotting Detection Reagent, chemiluminescent reaction reagent, was obtained from GE Healthcare. Enzyme linked immunosorbent assay (ELISA) kits for Interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) ware obtained from BioLegend (San Diego, CA, USA). Human recombinant soluble epoxide hydrolase (sEH) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA), and its substrate, *(2S,3S)-trans*-3-phenyl-2-oxiranylmethyl-4-nitrophenyl carbonate (S-NEPC), was from Sigma-Aldrich. Human recombinant chymase was obtained from Sigma-Aldrich, and its substrate, *N*-Succinyl-Ala-His-Pro-Phe-*p*-nitroanilide, was from Peptide Institute (Osaka, Japan). Hyaluronidase from bovine testes and Compound 48/80 were obtained from Sigma-Aldrich. Hyaluronic acid sodium salt from Rooster Comb was obtained from Wako Pure Chemical Industries.

HPLC analysis for quantification of hesperidin, neohesperidin, naringin, and naringenin

Prior to the sample preparation, dried *C. jabara* fruit peels were powdered. Amounts of hesperidin, neohesperidin, naringin, and naringenin were quantified as reported by Tosa *et al.*[15] with some modifications. Briefly, 50 mg of *C. jabara* powder were taken into a 10 mL vial and extracted with 50% methanol for 20 min using an ultrasonic extraction. Then, extract was passed through a 0.45 μm syringe

filter into a glass vial, and 5.0 µL was injected into the HPLC. Chromatographic separation was performed on a COSMOSIL 5C18-AR-II column (φ 4.6 x 250 mm) with a following condition: temperature, 40 °C; mobile phase, 20% acetonitrile containing 0.8% acetic acid; flow rate, 1.0 mL/min. Quantitative analysis was performed with purchased standard samples of hesperidin, neohesperidin, naringin, and naringenin. Peaks were detected at 280 nm. The limits of detection of each flavanone were follows: hesperidin, 40 ng/mL; neohesperidin, 92 ng/mL; naringin, 65 ng/mL; naringenin, 10 ng/ml, respectively. The quantification limits of each flavanone were follows: hesperidin, 120 ng/mL; neohesperidin, 280 ng/mL; naringin, 200 ng/mL; naringenin, 30 ng/mL, respectively.

Cell culture

The murine macrophage-like cell line, RAW 264 (RCB0535), was provided from the RIKEN BioResource Center (Ibaraki, Japan). RAW 264 cells were cultured in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 µg/ mL streptomycin. Cells were incubated at 37 °C with 5% CO_2 in a humidified atmosphere.

Cell viability assay

The cytotoxicity of flavonoids on RAW 264 cells were measured by Cell Counting Kit-8. Briefly, 1.0 x 10_5 cells/well of RAW 264 cells were seeded on a 96 well plate in DMEM with 10% FBS and antibiotics with various flavonoids at 37 °C for 24 h. Then, cells were washed, and added new 200 µL of DMEM with 10% FBS and 10 µL of WST-8 reagent. After further incubation for 2 h, cell viability was determined by measuring the absorbance at a wavelength of 450 nm. The cell viability ratio was calculated by the following formula: Cell viability (%) = (average of treated group/average of control group) x 100.

Measurement of nitric oxide (NO), IL-6, and TNF-α production

RAW 264 cells were seeded in a 96 well plate at a density of 1.0 x 10^5 cells/well in DMEM with 10% FBS and antibiotics at 37 °C for 2 h. Next, cells were cultured with or without flavonoids for 2 h, then, stimulated with 1 µg/mL of LPS for an additional 24 h. After treatment, culture supernatants were collected and stored -20 °C until assayed. The NO concentration was measured by using Griess reagent system. Average of LPS-induced NO production was 26.5 ± 1.4 µM. The concentration of IL-6 and TNF-α was measured by using mouse ELISA kits. Average of LPS-induced IL-6 protein was 1.034 ± 0.121 nM, and that of TNF-α protein was 5.030 ± 0.774 nM, respectively. The inhibitory ratio was calculated by the following formula: Inhibitory ratio (%) = (1-average of treated group/ average of control group) x 100.

Western blot analysis

RAW 264 cells were seeded in φ 60 mm dishes at a density of 6.0 x 10^6 cells/dish in DMEM with 10% FBS at 37 °C for 2 h. Next, cells were cultured with or without flavonoids for 2 h, then, stimulated with 1 µg/mL of LPS for an additional 24 h. After treatment, cells were washed and lysed with RIPA buffer and centrifuged at 20,600 xg for 30 min at 4 °C. Protein concentrations of supernatants were estimated by BCA protein assay kit, and stored at -80˚C until analysis. Equal amounts of protein (2.5 µg) of the cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. The membranes were pre-incubated in blocking solution at room temperature for 1 h. For iNOS protein detection, a membrane was incubated with a 1: 2,000 dilution of a primary antibody against iNOS at room temperature for 1 h. The blot was washed with T-PBS (-) and incubated with a 1:4,000 dilution of HRP-conjugated IgG secondary antibody. In case of β-actin, the transferred membrane was incubated with a 1:8,000 dilution of a HRP-conjugated antibody against β-actin at room temperature for 1 h. The target proteins on the membranes were detected using a chemiluminescent reaction and LAS-3000 Imaging System (Fuji film, Tokyo, Japan).

Enzyme inhibitory assay

sEH activity was measured as reported by Bahl *et al*.[16] with some modifications. Briefly, samples were incubated at 37 °C for 30 min in a 100 µL volume containing 0.58 U/mL sEH and 0.4 mM s-NEPC. Hyaluronidase activity was measured by the Morgan-Elson reaction as reported by Ratnasooriya *et al*.[17] Chymase activity was measured as reported by Tani *et al*.[18] with some modification. Briefly, samples were incubated at 37 °C for 60 min in a 100 µL volume containing 8.8 µg/mL chymase and 0.8 mM *N*-Succinyl-Ala-His-Pro-Phe-*p*-nitroanilide. These assays were carried out in 96-well plates and the optical density was measured at 405 nm in case of sEH and chymase, and at 585 nm in case of hyaluronidase, respectively. The inhibitory ratio was calculated by the following formula: Inhibitory ratio (%) = (1-average of increase in absorbance of sample/ average of increase in absorbance of control) x 100.

Results

Flavonoids in *C. jabara* fruit peels

Amounts of flavonoids in *C. jabara* fruit peels were quantified to clarify the distribution characteristics in *C. jabara* fruit peels. Eight flavones, 4 flavanones, and 3 chalcones were detected in *C. jabara* fruit peels, but more than 99% of flavonoids were narirutin (**Table 1**). Typical flavanone glycosides of *Citrus* fruit peels, such as hesperidin, neohesperidin, nor naringin, were not detected during the isolation and quantification process of *C. jabara* fruit peels. Furthermore, naringenin, an aglycone of narirutin, was also not detected.

For confirmation, amounts of hesperidin, naringin, neohesperidin, and naringenin in *C. jabara* fruit peels were quantified by HPLC analysis. Amounts of these flavonoids were below detection limit.

Toxicities of flavonoids in *C. jabara* fruit peels

Prior to investigate the anti-inflammatory effects, toxicities of purified flavonoids in *C. jabara* fruit peels were examined. Cell viabilities of RAW 264 cells cultivated with 100 and 200 μM flavonoids for 24 h were measured with Cell Counting Kit-8. In case of **FLV4**, toxicity of 200 μM was not evaluated because of its low solubility. At 100μM, no toxicity was observed on **FLV4**, narirutin, and its aglycone naringenin (**Figure 2**). Mild toxicity was observed on **FLV1-3** and **FNN2**. Strong toxicity was observed on **CHL1**. Toxicities of narirutin and naringenin were examined until 500 μM. Narirutin up to 500 μM and naringenin up to 400 μM exhibit no toxicity. At 500 μM naringenin, cell viability was 85.8 ± 6.0 %.

Flavonoids in *C. jabara* fruit peels inhibit LPS-induced NO production and iNOS protein generation in RAW 264 cells

In order to investigate the suppressive effects on NO synthesis and iNOS inhibitory activities, RAW 264 cells were cultivated with 1 μg/mL of LPS and 50, 100, and 200 μM of flavonoids for 24 h. NO concentrations in the culture supernatants were quantified by the Griess reaction. In case of **FLV4** at 200 μM, NO was not evaluated because of its low solubility. Expression levels of iNOS protein at 100 μM flavonoids were visualized by immunoblot analysis. All tested flavonoids, except for narirutin, exhibited inhibitory effects equally or more than that of L-NAME (**Figure 3A**). With a few exceptions, dose-dependently inhibitory effects were observed on each tested flavonoid. These flavonoids, except for narirutin, significantly reduced LPS-induced iNOS protein expression, but did not change β-actin expression (**Figure 3B**).

Flavonoids in *C. jabara* fruit peels inhibit LPS-induced IL-6 and TNF-α secretion in RAW 264 cells

In order to investigate the suppressive effects on IL-6 and TNF-α secretion, RAW 264 cells were cultivated with 1 μg/mL of LPS and 50, 100, and 200 μM of flavonoids for 24 h. IL-6 and TNF-α concentrations in the culture supernatants were quantified by the ELISA kits. In case of **FLV4** at 200

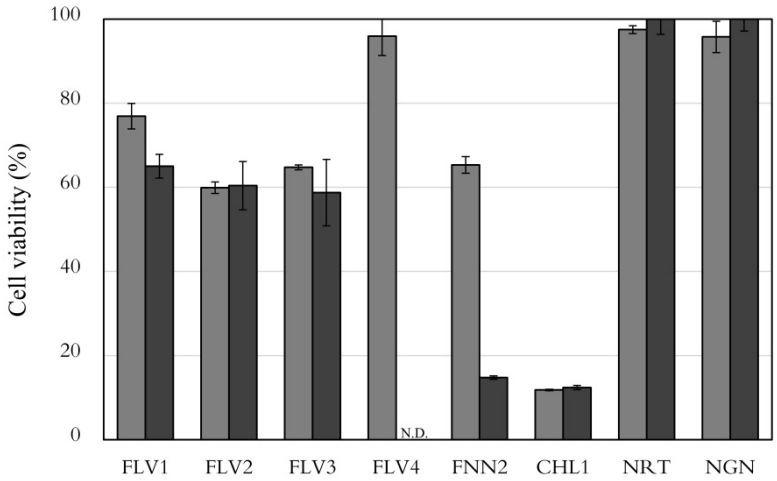

Figure 2. Toxicities of flavonoids in *C. jabara* fruit peels on RAW 264 cells. Flavonoids were tested at concentrations of 100 μM(■) and 200 μM(■). The values express the means ± S.D. of three experiments. NRT, narirutin; NGN, naringenin. Other abbreviations of flavonoids are listed in **Table 1**. N.D., no data.

μM, IL-6 and TNF-α were not evaluated because of its low solubility. Dose-dependently inhibitory effects were observed on each tested flavonoid. Among these flavonoids, **FNN2**, naringenin, and **FLV1** inhibited IL-6 secretion strongly, but narirutin and **FLV3** did weakly (**Figure 4A**). In case of TNF-α, only **FNN2** exhibited strong inhibitory activity (**Figure 4B**).

Inhibitory effects of narirutin and naringenin on inflammatory related factors

Inhibitory effects of narirutin and its aglycone naringenin on following inflammatory related factors were examined; secretions of NO, IL-6 and TNF-α in LPS-stimulated RAW 264 cells, and inflammation related enzymes, such as soluble epoxide hydrolase (sEH), hyaluronidase and chymase. Dose-dependent inhibitory effects were observed other than chymase (**Figure 5**). The inhibitory concentration 50% (IC_{50}) values of naringenin on inflammatory related factors were follows; NO production was 105 μM, IL-6 secretion was 65 μM, sEH was 267 μM, and hyaruronidase was 1,470 μM, respectively. On the other hand, narirutin did not exhibit significant inhibitory effects in the range of tested concentrations.

Discussion

In this study, we clarified the characteristics of flavonoids in *C. jabara* fruit peels, and investigated the anti-inflammatory effects of these purified flavonoids. Furthermore, we estimated the most

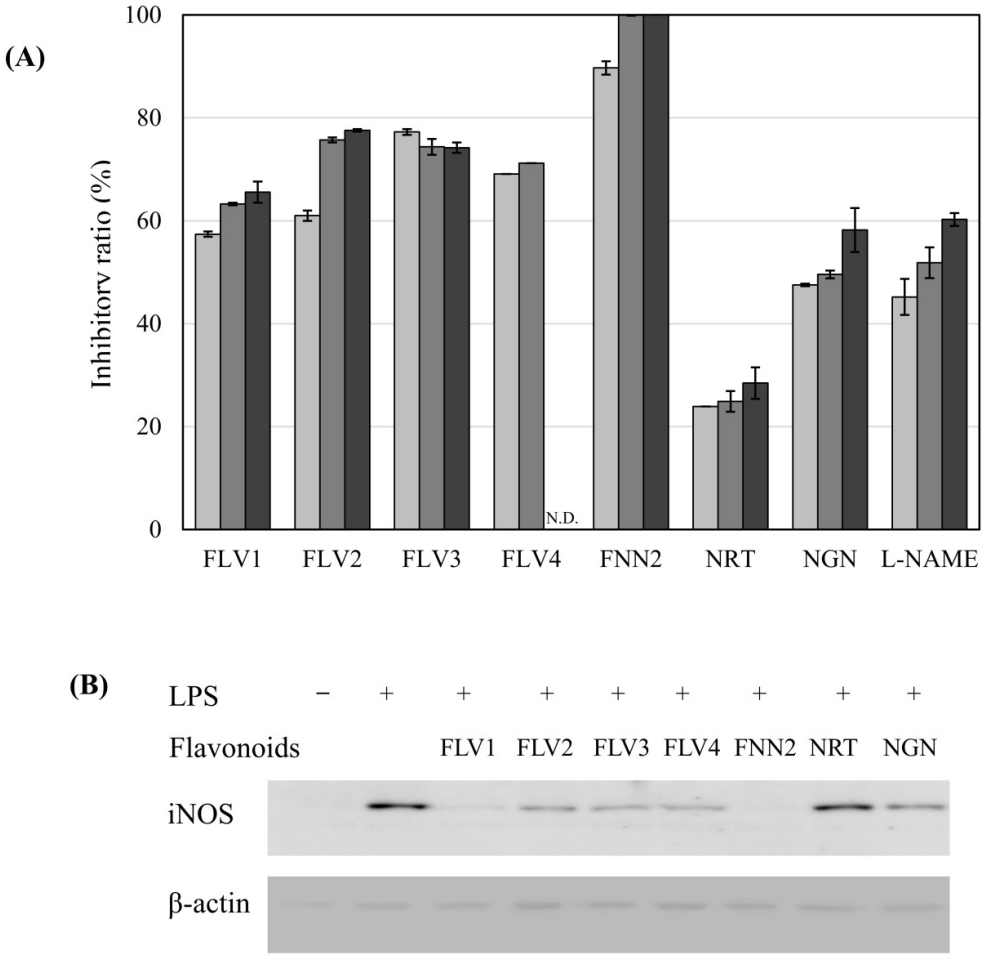

Figure 3. Flavonoids in *C. jabara* fruit peels inhibit LPS-induced NO production (**A**) and iNOS generation (**B**) in RAW 246 cells.

For NO production, flavonoids were tested at concentrations of 50 μM(■), 100 μM(■), and 200 μM(■). For iNOS generation, flavonoids were tested at concentration of 100.The values express the means ± S.D. of three experiments. NRT, narirutin; NGN, naringenin; L-NAME, N-Nitro-L-arginine methyl ester hydrochloride (positive control). Other abbreviations of flavonoids are listed in **Table 1**. N.D., no data.

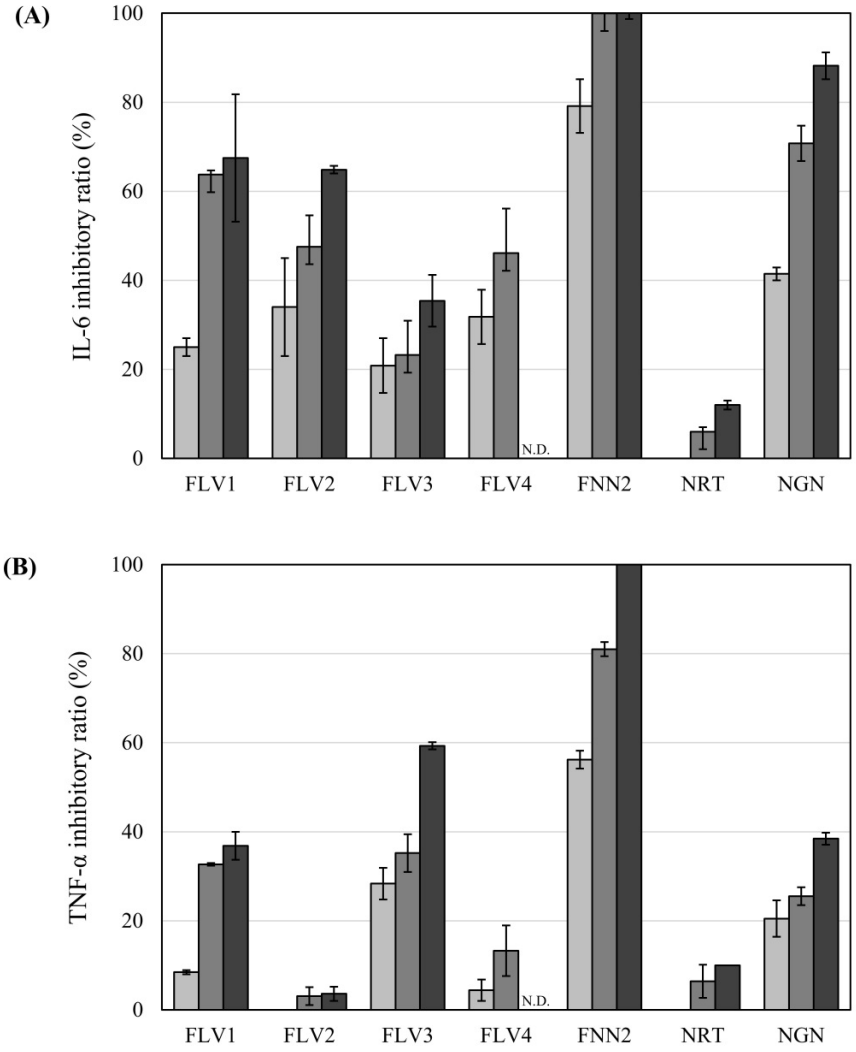

Figure 4. Flavonoids in *C. jabara* fruit peels inhibit LPS-induced IL-6 (A) and TNF-α (B) in RAW 246 cells. Flavonoids were tested at concentrations of 50 μM(■), 100 μM(■), and 200 μM(■). The values express the means ± S.D. of three experiments. NRT, narirutin; NGN, naringenin; L-NAME. Other abbreviations of flavonoids are listed in **Table 1**. N.D., no data.

effective anti-inflammatory compound in a living body.

Citrus could be classified by the content ratio of following 4 flavanone glycosides, such as narirutin, hesperidin, naringin, and neohesperidin, in its fruits[19]. Principal component analysis of *Citrus* fruits flavonoids indicated that distributions of *Citrus* species belonging to different classes were largely in accordance with Tanaka's classification system[20]. *C. jabara* is recognized as a closely-related species to *C. sphaerocarpa*, *C. sudachi*, *C. junos*, and *C. reticulate* by genetic characterization and diversity research[6–8]. As shown in **Table 1**, almost all of flavonoids in *C. jabara* fruit peels was narirutin, but hesperidin, naringin, and neohesperidin were below detection limit. There was no *Citrus* species with a flavanone glycosides content ratio like *C. jabara*[19,20]. This result indicates that *C. jabara* is a unique citrus.

Flavonoids in *C. jabara* fruit peels did not exhibit strong toxicity except for **CHL1** (**Figure 2**). Especially, narirutin and its aglycone naringenin were nontoxic. Thus, *C. jabara* fruit peel is considered as a nontoxic material, because almost all of flavonoids are narirutin in *C. jabara* fruit peels.

Anti-inflammatory effects of flavonoids were observed on inflammatory mediators in LPS-stimulated macrophage-like cells (**Figures 3 and 4**). Flavonoids are known as suppressors of nuclear factor κB (NF-κB) pathway[21,22]. Among

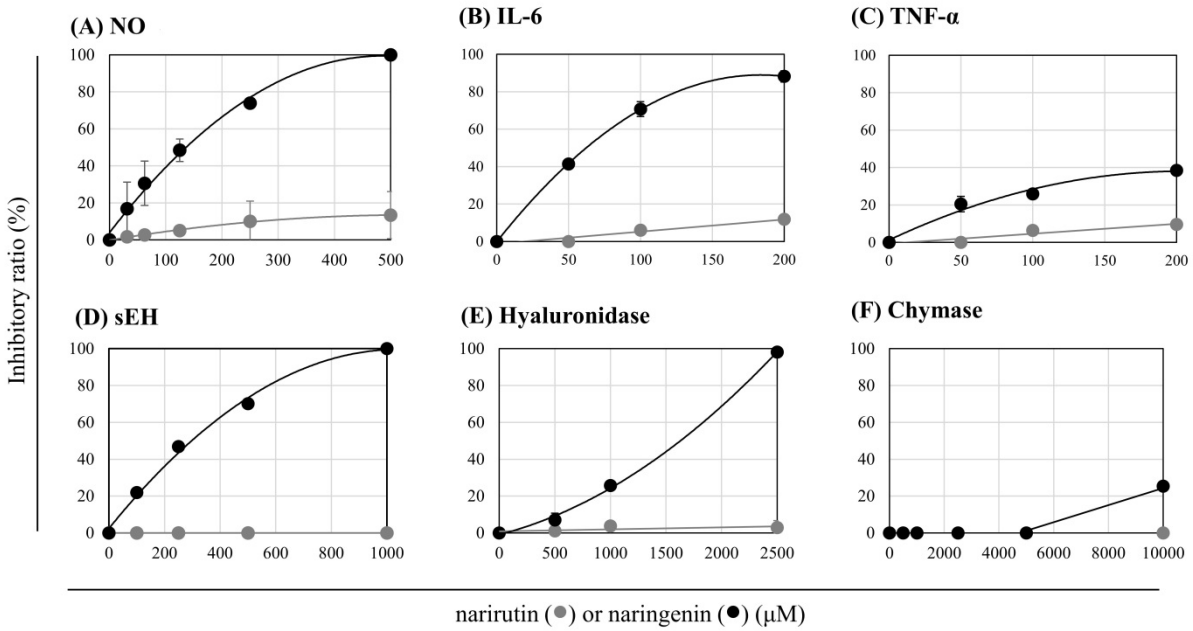

Figure 5. Inhibitory effects of narirutin and naringenin on inflammatory factors. (A)NO, (B)IL-6, (C)TNF-α, (D)sEH, (F)hyaluronidase, and (F)chymase were examined. The values express the means S.D. of three experiments.

tested flavonoids, **FNN2** was most effective on NO synthesis, iNOS induction, IL-6 synthesis, and TNF-α synthesis (**Figures 3** and **4**). These results may be effected by the reduction of cell viability, thus, toxicities of each flavonoid should also be taken into consideration. Although anti-inflammatory effects of narirutin was weak, but, its aglycone naringenin exhibited enough inhibitory effects (**Figures 3–5**). The difference in the inhibitory activity of narirutin and naringenin, in other word, glycosides and aglycone, may be partially explained by the difference in cell membrane permeability[23,24]. In this study, other structure-activity relations of inhibitory effects of tested flavonoids were not clear. For example, Takano-Ishikawa *et al*. reported that C2-C3 double-bond and 4-oxo functional group of the C-ring in flavonoids are important factors for the high inhibition activities of prostaglandin E_2 (PGE_2) induction by LPS in rat peritoneal macrophages[23]. In this study, all tested flavonoids have 4-oxo functional group in the C-ring. The C2-C3 double bond of C-ring, in short, flavone (**FLV**) skeletal structure, was not relevant to the inhibition activities in our results (**Figures 2–4**). **FNN2**, the most effective flavonoids, is one of most hydrophobic flavonoids in this study. But, the relationship between hydrophobicity of flavonoids and inhibition activities is considered not so high, because the inhibition activities of other most hydrophobic flavonoids, such as **FLV1** and **FLV4**, were not so high compare to other tested flavonoids.

Thinking about the abundance ratio of flavonoids, narirutin is considered to contribute to anti-inflammatory effect at oral ingestion of *C. jabara* fruit peels. Funaguchi *et al*. also reported that oral administration of narirutin inhibited airway inflammation in allergic model mouse[25]. On the other hand, *in vitro* cell assay, narirutin did not exhibited enough anti-inflammatory effects (**Figures 2–5**) nor anti-degranulating effects[26]. These conflicting results between *in vivo* and *in vitro* examinations may be explained by the metabolism of narirutin in a living body. Pharmacokinetics of narirutin after ingestion of orange juice in healthy humans were reported by Silveria *et al*.[27]. The majority of oral intake-narirutin is considered to reach the colon, and colonic microbiota deglycosylate narirutin to naringenin, that is then absorbed by colonocytes[28]. Thus, it is considered that narirutin exists like a prodrug, and its aglycone naringenin works as an active form within the body.

Anti-inflammatory effects of naringenin were also observed on inflammation related enzymes, such as sEH and hyaluronidase (**Figure 5D–5F**). sEH is known as a pro-inflammatory factor by metabolizing anti-inflammatory epoxyeicosatrienoic acids (EETs) to less potent dihydroxyeicosatrienoic acids (DHETs)[29]. Relationship of inflammation to hyaluronidase is also reported[30]. Therefore, naringenin is thought to work as an enzyme inhibitor, too. The wide difference of effective concentration of naringenin

was observed on each inflammatory related parameter (**Figure 5**), but this phenomenon is also observed in case of other flavonoids[31].

In this study, we clarified the large difference between narirutin and its aglycone naringenin on anti-inflammatory effects, and suggested the importance of deglycosylation of narirutin to naringenin at oral ingestion of *C. jabara* fruit peels. In fact, when patients with allergic disease intake *C. jabara* fruit peels, there are individual differences on anti-allergic and anti-inflammatory effects. Our findings may contribute to dissolve this problem. As shown in **Figure 1**, narirutin is a flavanone that contains rutinose group (Glc-Rham). Although hydrolysis of rhamnosides was not observed in human small intestinal cell extract[32], but rhamnosyl moiety of flavonoids could be hydrolyzed by strains of intestinal *Bacteroides* from humans[33]. Therefore, intestinal bacterial flora is considered as an important element for *C. jabara* fruit peels to exhibit anti-inflammatory effects. Now, we are trying to explore most suitable intestinal bacteria for deglycosylation of narirutin in *C. jabara* fruit peels in order to be more effective in more patients with allergic diseases.

Conclusion

C. jabara was a unique citrus that almost all of flavonoids in fruit peels were narirutin. Although anti-inflammatory effects of narirutin was weak, but, its aglycone naringenin exhibited following inhibitory effects: NO synthesis, iNOS induction, IL-6 synthesis, and sEH activity. Since narirutin is deglycosylated to naringenin that is then absorbed by colonocytes, it is considered that narirutin exists like a prodrug and its aglycone naringenin works as an active form of anti-inflammatory effect in a living body at oral ingestion of *C. jabara* fruit peels.

Acknowledgements

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Conflict of interest

The authors declare no potential conflicts of interest.

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CASE REPORT

Bullous pemphigoid induced by nivolumab in a patient with malignant melanoma

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ABSTRACT

Immune checkpoint inhibitors, such as nivolumab, have been recognized that the enhanced immune responses often lead to immune-related adverse events (irAEs) in various organs. Although cutaneous toxicity is one of the most common irAEs, bullous pemphigoid (BP) with immune checkpoint inhibitors is rare. Herein, the authors report a case of BP in a patient of the metastatic malignant melanoma of the brain under the treatment of nivolumab. It is notable that this case showed the clear correlation between the status of using of nivolumab and serum levels of anti-BP180 antibody. In addition, the skin eruptions in the case were mainly pruritic erosive or crusted papules and these clinical features may be the clinical characteristics of BP induced by nivolumab.

Keywords: *Immune-related Adverse Events (irAEs); Immune Checkpoint Inhibitors; Nivolumab; Bullous Pemphigoid (BP); Malignant Melanoma*

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Introduction

Immune checkpoint inhibitors utilizing monoclonal antibodies against programmed cell death protein-1 (PD-1) and programmed cell death ligand-1 (PD-L1), such as nivolumab, are new drugs for many types of solid malignancies[1,2]. These drugs increase antitumor immune responses and have been demonstrated as effective. However, the increased immune responses often lead to immune-related adverse events (irAEs) in many organs, including the skin. Recently, cases of bullous pemphigoid (BP) associated with nivolumab therapy have been reported, albeit rare. Herein, we report a case of BP induced by nivolumab.

Case presentation

A 67-year-old female, who was treated using nivolumab (3 mg/kg intravenously every 2 weeks) for the local recurrence of malignant melanoma of the brain for seven months, presented with pruritic erosive or crusted papules on erythematous bases, and a few vesicles on the arms, legs and trunk (**Figure 1**, **2**). Skin biopsy of the upper extremity revealed subepidermal vesicles with inflammatory cells (**Figure 3**). Direct immunofluorescence microscopy demonstrated linear deposition of IgG and C3 along the dermoepidermal junction (**Figure 4**). Indirect immunofluorescence using human skin with an artificial blister induced by 1 M NaCl incubation was positive for IgG against the epidermal side of the blister (**Figure 5**). On chemiluminescent enzyme assay, there were increased levels of serum anti-BP180 antibody (83.5 U/mL: normal < 9.0 U/mL). The titer of anti-desmoglein 1/3 antibody was below detection limit (< 3.0 U/mL). These findings were consistent with BP. We discontinued nivolumab, considering the possible association between nivolumab and BP, and started oral prednisolone (15 mg/day) and topical steroids. Skin lesions improved and serum

levels of anti-BP180 antibody rapidly decreased to normal; therefore, oral prednisolone was tapered. After five months, skin lesions were completely controlled and nivolumab was restarted. Pruritic erythematous papules reoccurred after three cycles of nivolumab and serum anti-BP180 antibody levels increased to 28.2 U/mL. As the tumor size gradually increased despite the administration of nivolumab, the chemotherapy was changed to dacarbazine. After the discontinuation of nivolumab, the skin eruptions rapidly improved and the serum level of anti-BP180 antibody decreased to within normal limits. Based on the clinical course, we diagnosed her with nivolumab-induced BP.

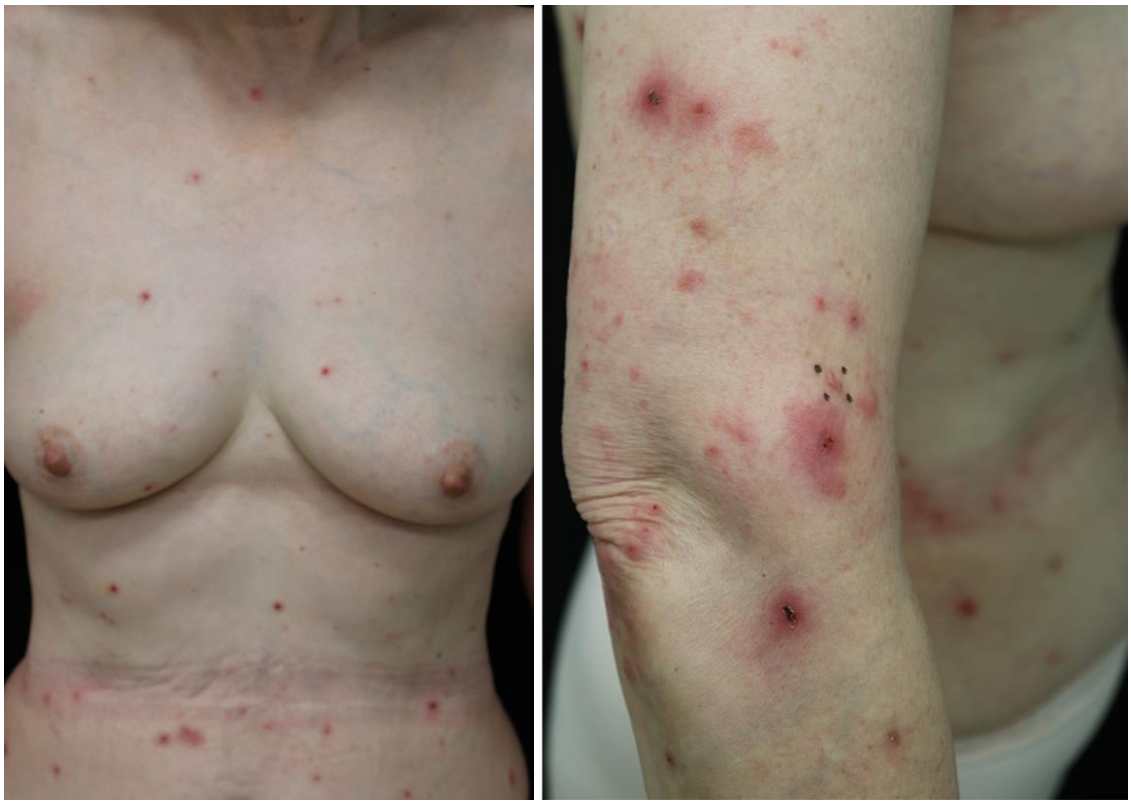

Figure 1, **2**. Pruritic erythematous papules and a few of vesicles on the arms, legs, and trunk. The black dots indicate the area of skin biopsy.

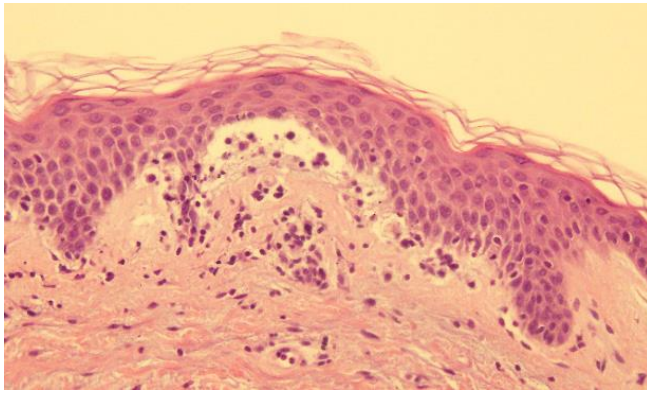

Figure 3. Histopathological findings of the skin lesion (hematoxylin and eosin staining, ×200).

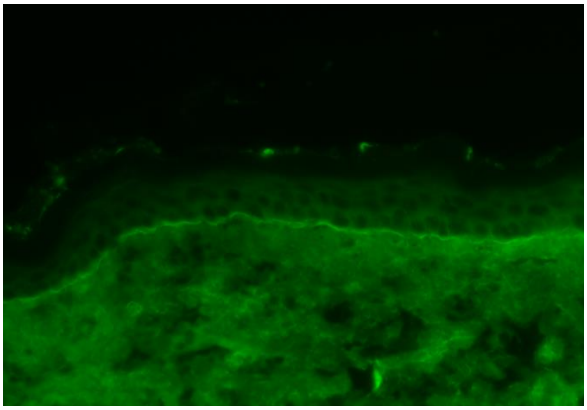

Figure 4. Direct immunofluorescence demonstrated linear deposition of IgG at the basement membrane zone.

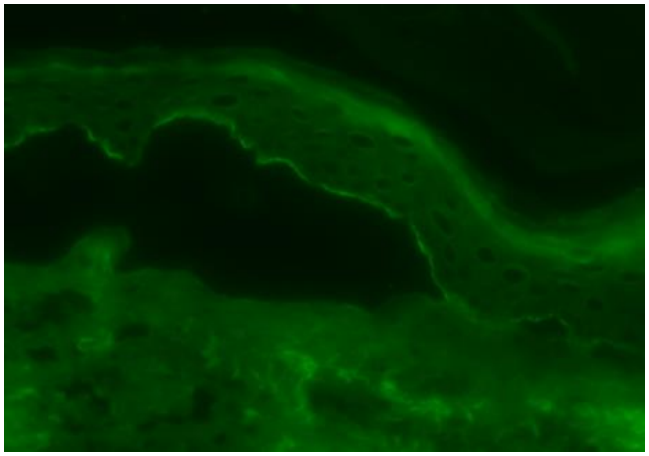

Figure 5. Indirect immunofluorescence was positive for IgG against the epidermal side of the blister.

Discussion

To our knowledge, BP is a rare cutaneous irAE and there are 16 previous cases of BP developing during nivolumab-therapy[3-7]. In oncologic patients, BP may be idiopathic, paraneoplastic or related to medication, including cancer therapy. Although it is generally difficult to distinguish immunotherapy-induced BP from other types of BP, a clear correlation between the status of using nivolumab and serum levels of anti-BP180 antibody was noted in our case, and we are able to exclude the possibility of idiopathic or paraneoplastic BP. Of note, the skin eruptions in our case were mainly pruritic papules, and these clinical features are similar to non-bullous type BP and may be the clinical characteristics of BP induced by nivolumab. Although the pathogenesis and clinical characteristics of BP induced by nivolumab have not been elucidated, clinicians should be aware of the possibility of BP associated with anti PD-1/PD-L1 antibodies and consider further examinations for BP even if vesicles or bullae are not found.

Conflict of interest disclosure

None declared

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MINI-REVIEW

Endocrine dysfunctions during treatment of immune-checkpoint inhibitors

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ABSTRACT

Immune-checkpoint inhibitors (ICIs) are novel agents directed to various malignant tumors. During ICI therapy, however, immune related adverse effects (irAEs) including endocrine dysfunctions have been reported. Dysfunctions in the pituitary gland and the thyroid gland by ICI are often observed, and those in the adrenal glands and the pancreas are less frequent. Positive correlation of the prevalence of endocrine irAEs to clinical antitumor effectiveness during ICI therapy has been reported. The mechanisms of endocrine irAEs by ICI, however, remain unclear, and optimal prevention, prediction, and treatment of the irAEs are still uncertain. This review describes possible mechanisms involved in ICI-related immunity, and discusses clinical management of endocrine irAEs during ICI therapy.

***Keywords**: PD-1; CTLA-4; immune-checkpoint inhibitors; endocrine organs; irAEs*

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Introduction

Immune-checkpoints play an indispensable role in anti-tumor immunity, anti-infection and autoimmunity[1,2]. Their major components include cytotoxic T-lymphocyte associated antigen 4 (CTLA-4), programmed death protein 1 (PD-1), and ligand for PD-1 (PD-L1) (**Figure 1, 2**). CTLA-4 is located on the surface of activated T-cells, therefore inhibiting binding of CD28 to B7 molecule on antigen presenting cells (APC)[1]. CTLA-4 pathway predominantly acts in lymph nodes. PD-1 is majorly expressed on T-cells[2]. PD-L1 is usually expressed on tumor cells, T-cells, B-cells, dendritic cells[2]. PD-1 pathway is involved with tumor microenvironment. APC or tumor cells present cancer-related antigen with major histocompatibility (MHC)-class I to T-cell receptor (TCR) on cytotoxic $CD8^+$ T-cell (**Figure 1**). Additionally, APC presents cancer-related antigen with MHC-class II to TCR on helper $CD4^+$ T-cell (**Figure 2**). $CD4^+$Th1-T-cells activate $CD8^+$ T-cells, and $CD4^+$Th2-T-cells stimulate antibody production. In antitumor immunity, $CD4^+$ T-cell and $CD8^+$ T-cell cooperate in the network.

Monoclonal antibodies to immune-checkpoints are referred to as immune-checkpoint inhibitors (ICIs), and are currently considered to be novel promising agents for treatment of malignant tumors[3,4]. ICI promotes T-cell mediated cytotoxicity directed to cancer cell antigens. It has been reported to improve prognosis of patients with malignant melanoma, renal cell cancer, non-small cell lung cancer, Hodgkin's lymphoma, and Merkel cell carcinoma. Approximately 20%-30% of patients with malignant melanoma, renal cell cancer, and non-small cell lung cancer were found to be responders of ICI. More than 50% of patients showed objective responses in the patients with refractory Hodgkin's lymphoma or Merkel cell carcinoma. On the other hand, various adverse events were reported as immune-related adverse

events (irAEs). IrAEs include dermatological, gastrointestinal, hepatic, neurological, and endocrine disorders[3,4] (**Table 1**).

In endocrine organs, irAEs in the pituitary gland, the thyroid gland, the parathyroid glands, the adrenal glands, the pancreas (type 1 diabetes mellitus) were reported[5-13]. Although ICI activates T-cells through inhibition of immune-checkpoints molecules, the etiology of endocrine irAEs remains unclear. Endocrine dysfunctions are sometimes crucial, and diagnostic procedures in endocrine dysfunctions are often complicated. Routine follow-up including physical examination with endocrinological investigation, as well as image testing is important. This review describes endocrine dysfunction during ICI therapy, and raises possible mechanisms involved with ICI-related immunity, highlighting diagnostic approaches and appropriate clinical management during ICI therapy.

Hypophysitis related to irAE

The pituitary gland is an endocrine organ central to regulate peripheral endocrine glands, by secreting several pituitary hormones; adrenocorticotrophic hormone (ACTH), thyrotropin (TSH), gonadotropin, growth hormone (GH), and prolactin, and antidiuretic hormone (ADH). The estimated prevalence of irAE related pituitary dysfunction is 9.1%[5], in contrast to idiopathic autoimmune hypophysitis which is only observed in 1 in 9 million individuals[14]. Incidence of irAE-related hypophysitis varies (0.4%-17.2%) in previous reports, the wide range associated with differences in surveillance[4,7,15,16]. Hypophysitis related to irAE occurs mostly within 6 months. In pituitary dysfunction, the incidence of secondary adrenal insufficiency: 'ACTH insufficiency', secondary hypothyroidism; 'TSH insufficiency', and secondary hypogonadism were 6.1%, 7.6%, and 7.5%, respectively[5]. The prevalence of GH insufficiency is not clear. Although rare, central diabetes insipidus due to pituitary posterior lobe dysfunction; 'ADH insufficiency' during ICI therapy was reported recently[17].

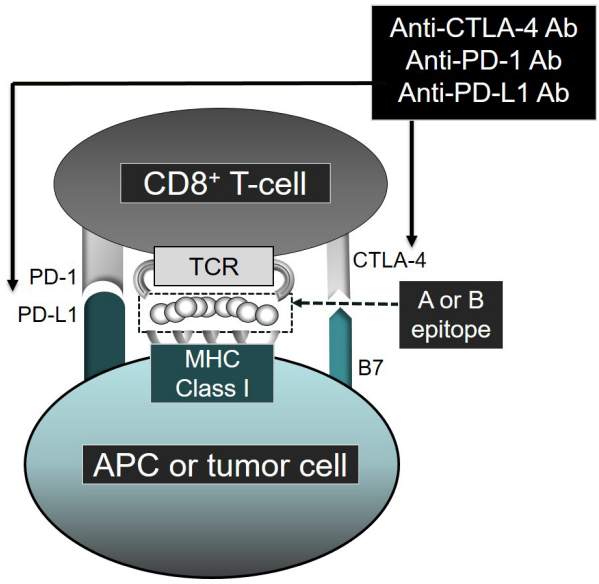

Figure 1. Relation of immune-checkpoints and $CD8^+$ T-cell is shown. A or B epitopes are presented on the surface of APC or tumor cell with MHC-class I to TCR on $CD8^+$ T-cell. Immune-checkpoint inhibitors regulate these immune reaction, and $CD8^+$ T-cell-mediated cytotoxic effect is enhanced.

A: tumor antigen, B endocrine organs specific antigens (*e.g.* thyroglobulin, insulin). APC: antigen presenting cell; TCR: T-cell receptor

Most of hypophysitis related to irAE is induced by anti-CTLA-4 antibody, and anti-PD-1 antibody–induced hypophysitis is less common (<1.0%) [4, 18]. Hypophysitis related to irAE occurs more often in men than women[15, 16]. In contrast, female predominance is seen in idiopathic autoimmune hypophysitis[14]. Common clinical manifestations in secondary adrenal insufficiency include headaches, fatigue, muscle weakness, and nausea, anorexia, weight loss, and hypotension are often reported[7,15,16]. In secondary hypothyroidism, constipation, fatigue, edema, and bradycardia can be seen. Manifestations in secondary hypogonadism and GH insufficiency can be not clear, but may be general fatigue and appetite loss. Notably, ACTH or TSH insufficiency are the most common pituitary hormone abnormalities in hypophysitis related to irAE [15,16].

Secondary adrenal insufficiency is diagnosed with decreased ACTH and cortisol, and severe cases show hypoglycemia, hyponatremia, and hyperkalemia. Secondary hypothyroidism is diagnosed with decreased FT3, FT4, TSH, and sometimes hyponatremia, and elevated CK or cholesterol are seen. Secondary hypogonadism was diagnosed with decreased gonadotropin with decreased sex hormones (estradiol or testosterone). Low levels of GH and insulin-like growth factor-1 (IGF-1) are seen in GH insufficiency. In imaging tests, pituitary enlargement is sometimes seen in hypophysitis related to irAE in pituitary MRI[15,16]. Incomplete secretion of ADH and disappearance of T1-weighed high signal in posterior lobe in pituitary MRI is observed in central diabetes insipidus[17]. In order to precisely diagnose, pituitary provocation tests is often recommended. ACTH insufficiency is usually permanent, on the other

Table 1. Summary of endocrine dysfunctions during immune-checkpoint inhibitors treatment

Affected organ	Diagnosis	Prevalence		Onset	Major symptoms	Laboratory data	Imaging tests	Recommended managements	Additional requirements
Pituitary gland	Secondary adrenal insufficiency	6.1%	9.1%	Mostly within 6 months	Fatigue, muscle weakness, nausea, anorexia	ACTH↓cortisol↓	With/without pituitary swelling in pituitary MRI	Hydrocortisone replacement	1) Pituitary provocation tests, and ACTH infusion test is often recommended. 2) Higher dosage of prednisolone is used for severe persistent headache or visual symptoms. 3) Adrenal insufficient should be firstly treated if hypothyroidism is concomitant.
	Secondary hypothyroidism	7.6%	Depend on each report (0.4-17.2%)		Constipation, fatigue, edema, bradycardia	FT3↓FT4↓TSH↓		L-T4 replacement	
	Secondary hypogonadism	7.5%	Mostly with anti-CTLA-4 Ab, and uncommon with anti-PD-1 Ab (<1%)		General fatigue appetite loss	Gonadotropin↓		Gonadotropin replacement if needed	
	GH insufficiency	Not determined				GH↓IGF-1↓		GH replacement, if needed	
	Central diabetes insipidus	Not determined		Not clear	Polydipsia and polyuria	Serum osmolality↑ADH↓	Disappearance of T1-weighed high signal in posterior lobe in pituitary MRI	Desmopressin acetate (DDAVP)	
Thyroid gland	Thyrotoxicosis (destructive thyroiditis) (often)	9% with anti-PD-1 Ab 0-4% with anti-CTLA-4 Ab		2-6 weeks, mostly within 6 months	Palpitation, weight loss, tremor, fatigue, diarrhea	FT3↑FT4↑TSH↓	Color Doppler flow↓ $^{123}I/^{99m}Tc$ uptake↓	Beta-blockers may be used	
	Thyrotoxicosis (hyperthyroidism) (rare)	Depend on each report (0-39.1%)				FT3↑FT4↑TSH↓ TRAb(+)	Color Doppler flow↑ $^{123}I/^{99m}Tc$ uptake↑	Anti-thyroid drug, ^{131}I treatment, or thyroid surgery	
	Hypothyroidism				Constipation, fatigue, edema, bradycardia	FT3↓FT4↓TSH↑	Color Doppler flow↓ $^{123}I/^{99m}Tc$ uptake↓	L-T4 replacement	Adrenal insufficiency should be firstly treated if hypothyroidism is concomitant.
Adrenal gland	Primary adrenal insufficiency	1%		Mostly within 1-6 months	Fatigue, muscle weakness, nausea, anorexia	ACTH↑cortisol↓	With/without adrenal swelling in abdominal CT	Hydrocortisone replacement	
Pancreas	Type 1 diabetes mellitus	0.6%		Mostly within 1-6 months	Thirst, polydipsia, and polyuria	plasma glucose↑ HbA1c levels are not always elevated.	With/without pancreas swelling in abdominal CT	Insulin therapy	

hand, secondary hypothyroidism/hypogonadism may recover. Positivity of anti-pituitary antibody may be helpful for diagnosis of hypophysitis related to irAE[18,19]. As predictive factors for hypophysitis related to irAE, eosinophilia, elevation of ESR/CRP/LDH, and hyponatremia can be useful[9].

In monitoring and treatments, baseline and follow-up thyroid function (serum levels of FT3, FT4, and TSH) and morning ACTH and cortisol are crucial, because imbalance of hypothalamus-pituitary-thyroid (H-P-T) axis or hypothalamus-pituitary-adrenal (H-P-A) axis is life-threating. A cosyntropin (; synthetic ACTH), infusion test is helpful to distinguish primary or secondary (pituitary-originated) adrenal insufficiency. Levels of GH-IGF-1, gonadotropin-sex hormones, and prolactin, should be assessed in patients with hypophysitis or hypopituitarism. High-dose steroids (*e.g.* 1-2 mg/kg/day of oral prednisolone) is used as treatment for those patients with severe headache, or visual abnormalities[15,16]. In cases of critical hypophysitis or hypopituitarism, intravenous glucocorticoid infusion is considered. Otherwise, oral hydrocortisone (15-30 mg/day) is used for treatment of adrenal insufficiency. In cases of adrenal crisis, higher dose of hydrocortisone (300 mg/day) is administrated intravenously. Levothyroxine (L-T4) (typically starting from 25 μg/day with increasing dosage) is used for secondary hypothyroidism; but adrenal insufficiency should be treated first to avoid adrenal crisis induced by increasing thyroid hormone effect. In cases of secondary hypogonadism or GH insufficiency, gonadotropin replacement or GH replacement therapy can be considered, respectively. In cases of central diabetes insipidus, Desmopressin acetate (DDAVP) is used[17].

Thyroiditis related to irAE

The thyroid gland is an organ to produce thyroid hormone (T3, T4), and controls systemic metabolism. Major autoimmune thyroid diseases (AITD)s are Graves' disease (GD) in which TSH receptor is autoantigen (20), and Hashimoto's thyroiditis (HT) in which TPO and Tg are autoantigen[21]. Cell-mediated immunity and antibody-dependent cellular cytotoxicity (ADCC) are involved in AITD. An interim analysis of Japanese patients with malignant melanoma underwent ICI therapy showed high prevalence of thyroid disorders (any grade; 23.53%, and ≥Grade 3; 0.44%)[22]. In contrast to pituitary irAEs, the thyroid gland is preferentially affected by anti-PD-1 antibody rather than anti-CTLA-4 antibody. Thyroiditis related to irAE occurs in 0-4% patients treated with anti-CTLA-4 antibody[7]. Prevalence of thyroiditis by anti-PD-1 antibodies is estimated to be 9%[6]. IrAE-associated thyrotoxicosis and hypothyroidism similarly prevalent[6,23,24]. Transient thyrotoxicosis may precede hypothyroidism[23,24]. Dual treatment of nivolumab and ipilimumab induces thyroiditis in 22% of patients[25]. More recent reports showed increased incidence of anti-PD-1 antibody-induced thyroid dysfunction in 17.1% of malignant melanoma patients[24] and 21% of non-small cell lung cell cancer patients, respectively[26]. Morganstein, et al reported that ipilimumab-induced thyroid dysfunction occurred in 23% of patients, anti-PD-1-induced thyroid dysfunction in 39.1% of those, and dual treatment induced thyroid dysfunction in 50% of those[27]. Anti-PD-L1 antibody treatment was reported to induce 2-4% of thyroid dysfunction[28]. Patients who have anti-TPO-antibody (TPOAb) or anti-Tg-antibody (TgAb) are reported to be prone for development of thyroiditis related to irAE[29]. Thyroiditis related to irAE can occur early; between 2 and 6 weeks and mostly within 6 months after treatment. Notably, a proportion of the ICI-treated patients exhibit `non-thyroidal illness syndrome` (normal FT4, normal TSH, and decreased FT3), reflecting lower nutrition status. Thyroid dysfunction during pembrolizumab treatment was reported to have possible association with feasible outcome of lung cancer[29].

Thyrotoxicosis: thyrotoxicosis related to irAE is classified into two subtypes; 1) destructive thyroiditis, and 2) hyperthyroidism manifested as GD. Palpitation, weight loss, tremor, fatigue, and diarrhea are typically seen as thyrotoxic symptoms.

1) Destructive thyroiditis: destructive thyroiditis is often induced by ICI, transient increase of FT3 and FT4 are seen, concomitant with suppressed TSH levels. Anti-TSH receptor antibody (TRAb) is negative in most of the cases. TPOAb or TgAb are often positive at diagnosis[23,24]. Elevation of serum Tg levels may help the diagnosis of thyroiditis[30]. In thyroid ultrasonography, Doppler blood flow is not increased, thyroid uptake in ^{123}I or ^{99m}Tc scintigram is usually decreased, and FDG-PET is positively accumulated in the thyroid[24]. Diffuse thyroid enlargement can be seen[29]. In treatment, β-blocker is effective for relieving thyrotoxic symptoms. Effectivity of steroid treatment is not clear.

2) GD induced by ICI: Rare cases of thyrotoxicosis show elevated FT3 and FT4 concomitant with suppressed TSH, and positivity of TRAb, suggesting GD during ICI treatment[31]. In those cases, increased color Doppler flow and increased uptake in ^{123}I or ^{99m}Tc scintigram were seen,

and treatment options are anti-thyroid drug, ^{131}I treatment, or thyroid surgery.

Hypothyroidism: Hypothyroidism related to irAE is classified into two subtypes; 1) transient thyrotoxicosis followed by hypothyroidism, and 2) a single phase of hypothyroidism[6,23,24]. Hypothyroidism is due to thyroiditis by ICI. Patients who have TgAb or TPOAb are prone to be hypothyroidism[29]. Primary hypothyroidism is diagnosed by decreased FT3 and FT4, and increased TSH. Primary hypothyroid symptoms are similar to those in secondary hypothyroidism mentioned above. In thyroid ultrasonography, Doppler blood flow may be decreased, and thyroid uptake in ^{123}I or ^{99m}Tc scintigram is decreased. Diffuse thyroid swelling is seen in acute phase of thyroiditis, and atrophy can be seen in subsequent hypothyroid phase[29]. L-T4 (typically starting from 25 μg/day with increasing dosage) is used to treat hypothyroidism. Cases of hypothyroidism often progress to permanent hypothyroidism[23]. In monitoring, baseline measurement of thyroid autoantibodies (TRAb, TgAb, and TPOAb), and routine checkup of serum FT3, FT4 and TSH levels are recommended. Eosinophilia, thrombocytopenia, ESR/CRP/LDH elevation, and liver dysfunction might be important for earlier detection of thyrotoxicosis[9].

Adrenalitis related to irAE

Adrenal glands secrete adrenal hormones to maintain systemic activity. Cortisol is one of the critical adrenal hormones, regulating glucose metabolism, blood pressure, and other homeostasis. Cases of autoimmune adrenalitis were reported, with presence of autoantibodies to P450c21 or P450c17; adrenal enzyme involved with adrenal hormone synthesis[32]. Adrenal failure is due to incomplete secretion of adrenal hormones including cortisol. IrAE-associated adrenal hypofunction is classified to primary or secondary adrenocortical insufficiency. Primary adrenocortical insufficiency is due to adrenalitis[10], and secondary adrenocortical insufficiency is due to pituitary damage described above[5]. Prevalence of adrenalitis related to irAE is reported as approximately 1%[33]. Adrenal dysfunction usually occurs within 1-6 months after initiation of ICI[10, 34-36]. Either anti-PD-1 antibody[10,34] or anti-CTLA-4 antibody[35,36] induces adrenalitis.

Symptoms in primary adrenal insufficiency are almost the same as those seen in secondary adrenal insufficiency. Decreased serum cortisol, increased plasma ACTH, and decreased urine cortisol levels indicate diagnosis of adrenalitis. In an ACTH stimulation test, response of cortisol is lost in cases of primary adrenal insufficiency. Presence of autoantibodies to P450c21 or P450c17 may be useful for diagnosis of adrenalitis. As a treatment, oral hydrocortisone replacement therapy (15-30 mg/day) is used. In cases of adrenocortical crisis, higher dose of hydrocortisone (300 mg/day) are administrated intravenously.

Type 1 diabetes mellitus and other endocrine dysfunctions related to irAE

Pancreatic beta cells produce and secrete insulin. Insulin controls blood glucose levels. In patients with type 1 diabetes (T1DM), pancreatic beta cells are destroyed, and insulin secretion is attenuated. Insulin is considered to be an autoantigen in T1DM. As a pancreatic irAE, patients with T1DM during ICI therapy were reported[11,12,37]. Prevalence of T1DM associated with ICI therapy was reported to be 0.6%[37]. Anti-PD-1 antibodies more often induce T1DM than anti-CTLA-4 antibodies. As a severe subtype of T1DM, fulminant type 1 diabetes (F1DM) was also reported. T1DM related to irAE occurs mostly within 1-6 months after ICI treatment, and often rapidly progressing with ketoacidosis. T1DM related to irAE is thus one of the urgent complications of irAE.

Thirst, polydipsia, and polyuria are seen as hyperglycemic symptoms, and elevated plasma glucose is seen. HbA1c levels are not always elevated, reflecting acute development of hyperglycemia. Sometimes swelling of pancreas is seen in abdominal CT. Anti-GAD, anti-IA2, and anti-insulin autoantibodies are sometimes positive. Immediate insulin therapy is required in patients with T1DM related to irAE. Monitoring of plasma glucose and HbA1c are useful for detection of T1DM. Measurement of anti-GAD antibody is also helpful to find individuals who may be susceptible to T1DM. Although rare, hypoparathyroidism related to irAE was reported as an endocrine irAE[13].

Possible mechanisms in endocrine irAEs

Multiple factors influence in the development of endocrine-related irAEs. In certain genetic and environment backgrounds, ICI may activate immune systems especially autoreactive cytotoxic $CD8^+$ T-cells directed to endocrine organs [2].

Genetic factors of immune-checkpoints in irAEs

Altered function or genetic mutation of immune-checkpoint molecules may contribute to the development of endocrine irAEs. In AITD, genetic mutations in CLTA-4 in GD[38] and HT[39] were reported. An association of a SNP in PD-L1 with GD was shown[40]. SNPs in CLTA-4 and PD-L1 also contribute to the development of autoimmune adrenalitis[41]. PD-L1 and PD-1 genetic mutation are reported to be involved in T1DM development[42,43].

Molecular mimicry of tumor antigen and auto-antigen in endocrine organs

One of the tumor-associated antigen: NY-ESO-1 possess common amino acid sequence with thyroid autoantigens (TSH receptor, Tg, and TPO), and administration of NY-ESO-1 was reported to induce GD or HT in individuals who have risk allele of MHC (class I or class II)[44]. In a case of malignant melanoma with F1DM during ICI treatment, HLA-DR4 restricted insulin autoantigen with positive conversion of anti-insulin antibody was suggested[11]. Therefore, upon both activation of anti-pancreatic beta cells and anti-melanoma immunity, exacerbation of pancreatic beta cell function and improvement of malignant melanoma can be seen in patients with F1DM related to irAE. Distinct manifestations of irAE inpatients with various malignant diseases were reported (*e.g.* malignant melanoma patients had a higher frequency of gastrointestinal/skin irAE, and a lower frequency of pneumonitis related to irAE)[45]. Thus, various tumor epitope may possess common amino acid sequences with endocrine epitope (*e.g.* TSH receptor, Tg, TPO, insulin). And cross-presentation of those on HLA molecules may be related to endocrine irAEs (**Figure 1,2**).

Inhibition the role of immune-checkpoints in endocrine irAEs

Anti-CTLA-4 antibodies often induce auto-immunity, by inhibiting regulatory T-cells function, and by activation of immunogenic $CD4^+$T-cells and $CD8^+$T-cells[46,47]. In animal models, anti-CTLA-4-antibodies were reported to induce thyroiditis[48], adrenalitis[49], and T1DM[50]. Anti-PD-1 antibodies blocks PD-1 pathway which suppress autoimmunity. Subsequently, immunogenic $CD4^+$T-cells and $CD8^+$T-cells can be activated. PD-1 knockout mice showed lupus-like autoimmune disorders[2], and PD-1 blockade was reported to induce T1DM in mice[51].

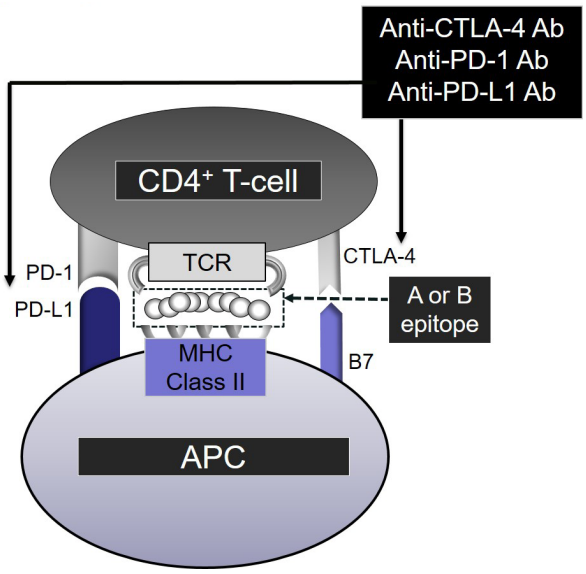

Figure 2. Relation of immune-checkpoints and $CD4^+$ T-cell is shown. A or B epitopes are presented on the surface of APC with MHC-class II to TCR on $CD4^+$ T-cell. Immune-checkpoint inhibitors regulate these immune reaction, and $CD4^+$ T-cell is activated.

A: tumor antigen, B endocrine organs specific antigens (*e.g.* thyroglobulin, insulin). APC: antigen presenting cell; TCR: T-cell receptor

Other possible mechanisms in endocrine irAEs

Higher expression of CTLA-4 in the pituitary gland was reported to be related to hypophysitis, due to type II or type IV allergy by IgG1 or IgG2 type anti-CTLA-4 antibodies[18]. Yamauchi, *et al*. reported that PD-L1 and PD-L2 expressions on thyroid gland might be related to nivolumab-induced thyroiditis[52].

Discussion

To date, most authors have recommended both discontinuation of ICIs and high dose glucocorticoid for treatment of irAEs. However, this has been challenged by recently, particularly if the endocrine irAEs can be managed, and the anti-tumor therapy is effective[53]. In addition, correlation of prevalence of irAEs to anti-tumor immunity was recently shown[54]. It is of interest to examine whether bio-markers which have been reported in malignant melanoma can be applied to the patients with endocrine irAEs[55]. Further investigations with larger number of cases over longer periods are warranted to establish diagnostic and therapeutic approaches to endocrine irAEs. In the future, identification of individuals (*e.g.* genetic predispositions such as HLA type, age, gender) who are susceptible to irAEs are necessary[3,4].

Conflict of interest

The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of their article.

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MINI-REVIEW

Serological biomarkers of granuloma progression in sarcoidosis

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ABSTRACT

Sarcoidosis is a systemic disorder with unknown etiology and pathogenesis characterized by non-caseating granulomas, and different clinical manifestations of sarcoidosis hinder diagnosis and treatment. Therefore, a comprehensive understanding of serological markers based on clinical observations of sarcoidosis and the progression of granulomas would aid analysis in routine clinical practice. In this review, we overview common serological markers, including angiotensin converting enzyme (ACE) and lysozyme, and describe in detail new promising indices in sarcoidosis such as a T cell serological marker (soluble interleukin 2 receptor; sIL-2R) and thymus and activation-regulated chemokine (TARC/CCL17).

***Keywords**: serological biomarkers; angiotensin converting enzyme; lysozyme; soluble interleukin 2 receptor, granuloma; sarcoidosis*

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Introduction

Sarcoidosis has striking clinical heterogeneity, but the etiology and pathogenesis of sarcoidosis are still poorly understood[1-4]. Various clinical manifestations ranging from no symptoms to severe outcomes, such as respiratory dysfunction, blindness, severe neurological disorders, and cardiac life-threatening conditions, may hinder diagnosis and treatment of sarcoidosis[4].

Diagnosing sarcoidosis is problematic because there is no definitive test[4]. Diagnosis is mainly based on a combination of clinical, radiological, and serological features, and supported by pathological findings[5]. Therefore, evidence of non-caseating granulomas from an easily accessed organ, such as a cutaneous tissue biopsy, facilitates diagnosis. The clinical utility of common serological markers is based on correlations with clinical manifestations, dynamics of the disease, and other severity indices such as pulmonary radiographical stage, the number of $CD4^+$ T cells in bronchoalveolar lavage fluid (BALF), and the number of affected organs imaged by FDG ^{18}PET or ^{67}Ga scintigraphy[6]. However, there are no useful diagnostic, prognostic, or therapeutic serological markers that support the management of sarcoidosis in patients[4].

Sarcoidosis is a systemic disorder characterized by non-caseating granulomas. Granulomas are a collection of monocyte-macrophage lineage cells and lymphocytes, which surround but cannot eliminate various pathogens such as microorganisms, toxic molecules, or foreign bodies[7,8]. Granulomas containing pathogenic agents are "seeds" and are surrounded by epithelioid cells, mononuclear cells, and T cells on the periphery (**Figure 1**). These cells engulf or trap the pathogenic agents and prime the immune system by releasing pro-inflammatory cytokines[4]. The distribution of the cell population and intensity of the immune reaction may help shape acute or chronic disease

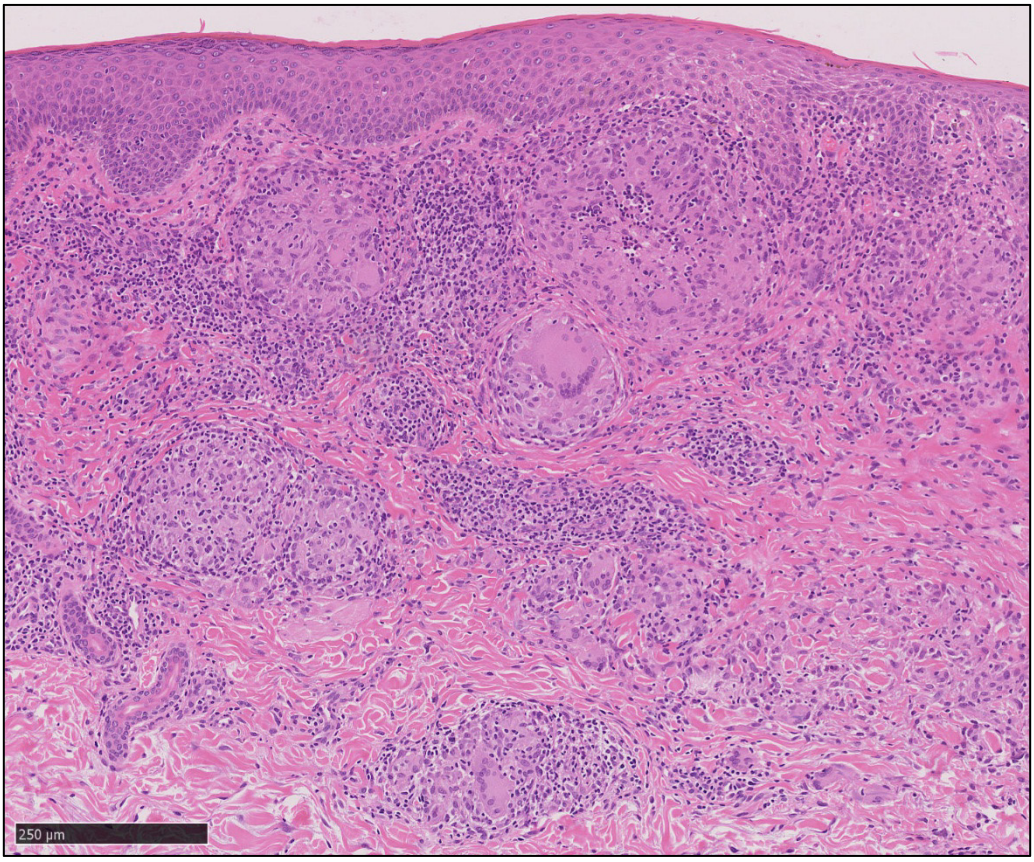

Figure 1. Network of cells in the formation of sarcoidal granuloma. A biopsied skin tissue with HE staining shows multiple non-caseating granulomas in the dermis. In sarcoidosis, a network of cells is responsible for building granulomas. The compact structure contains abundant epithelioid cells, multinucleated giant cells, and peripheral inflammatory cells, which are mainly T cells. Magnification is 20x.

activity[9].

The origin of secreted serological markers during granulomatous inflammation may help track clinical progression of the disease. Here, we outline some common serological markers and focus on some promising T cell markers in sarcoidosis. In addition, we comprehensively describe the relationship between serological markers and the stages of sarcoidal granuloma formation, which may aid analysis of serological tests in routine clinical practice (**Figure 2**).

Serological markers associated with granuloma

At the "mature" stage of sarcoidal granuloma structure, there is a compact and organized collection of cells with abundant epithelioid cells at the center[3]. These cells may fuse together and form multinucleated giant cells. Monocyte-macrophage lineage cells in the granuloma secrete ACE and lysozyme, which are commonly used "traditional" indices of total granuloma mass in sarcoidosis.

Epithelioid cells are the primary sources for ACE, and ACE is a measure of granuloma burden in sarcoidosis[3,10,11]. However, the role of ACE in the diagnosis of sarcoidosis is controversial because of low sensitivity and specificity[12]. The sensitivity of ACE levels for the diagnosis of sarcoidosis is 40–60%[13-16], but may be as low as 29%[6], which is similar to a study by Turton *et al*.[16] **Table 1** outlines the factors that contribute to the sensitivity and specificity of serum ACE. One factor that affects the sensitivity of ACE is an insertion (I)/delete (D) polymorphism in the ACE gene, which results in significantly different serum ACE levels between genotypes DD (high serum ACE), ID (intermediate serum ACE), and II (low serum ACE)[17]. In addition, ACE is a secondary feature of sarcoidosis rather than an initial event of monocyte-macrophage lineage activity[18]. ACE is released by epithelioid cells, which are polarized by monocyte-macrophage lineage cells at the mature building stage of granuloma (**Figure 2**)[3]. Therefore, ACE may not be sensitive at an early stage and is probably not a good index of disease activity. Nevertheless, serum ACE

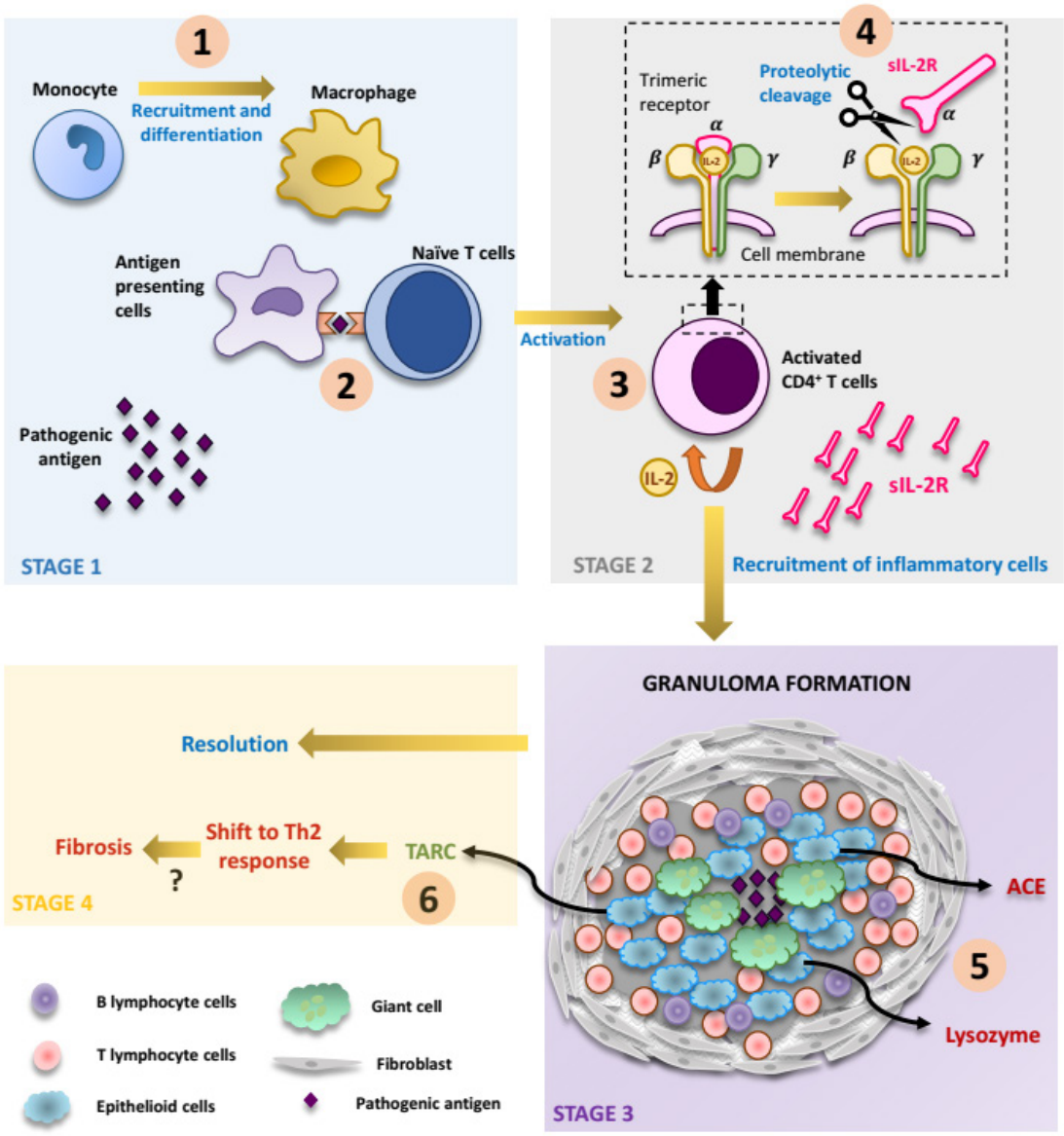

Figure 2. Serological markers during different stages of sarcoidal granuloma formation. Granuloma formation occurs in 4 stages including stage 1 (initial stage), stage 2 (accumulation of inflammatory cells), stage 3 (effector phase with the amplification of immune response to create a compact cell network to form granuloma), and stage 4 (resolution or fibrosis progression), and T helper cells are clearly involved in the mechanism of initiation, maintenance, and progressive fibrosis[3]. The main events that explain the expression of serological biomarkers during this progression are as follows:
(1) First, pathogenic antigens, including microorganism, toxic molecules, or foreign bodies[7,8], activate the innate immune system and recruit peripheral monocytes, which then undergo differentiation into antigen-presenting cells.
(2) Antigen presentation activates naïve T cells.
(3) Activated T cells produce IL-2 as a local growth factor for T cells[5] and express IL-2 receptor on their surface.
(4) Proteolytic cleavage of IL-2α chain occurs after T cell stimulation[27]. The presence of the α chain in body fluids is a good measure of T-cell activation.
(5) Immune granulomas have a central follicle composed of a pathogenic antigen and epithelioid cells. These cells are the main source of ACE and lysozyme. T cells and B cells surround the "granuloma core" and are encircled by fibroblasts.
(6) TARC is mainly secreted by epithelioid cells and may drive the Th2 immune response in sarcoidosis. Shifting to a Th2 response may favor fibrotic progression and further investigation is needed to elucidate the underlying mechanism of this phenomenon.

Table 1. Reasons for the poor sensitivity and specificity of serum ACE levels in the diagnosis of sarcoidosis.

1. Differences in test assays
2. Differences in abnormal cut-off values
3. Inconsistent diagnoses of sarcoidosis
4. Measurement of serum ACE levels at different time points
5. ACE may change after treatment[38]
6. Polymorphism of the ACE gene[17]

levels greater than two times the upper limit of the normal range can be used to support diagnosis and assessment of the clinical course of the disease[12,19].

Lysozyme is a bacteriolytic enzyme produced by activated macrophages and epithelioid cells in the sarcoidal granuloma, but is not detected in regressing lesions[20]. Serum lysozyme levels increase in sarcoidosis, but its diagnostic value is poor[12,21]. About 70% of patients with sarcoidosis have a high level of serum lysozyme, but the sensitivity of lysozyme is only 26% in sarcoidosis patients with cutaneous lesions[16]. Lysozyme may be a marker of pulmonary lesions because it correlates with chest radiological stages and the number of total cells, lymphocytes, and $CD4^+$ T cells in BALF[20]. Like ACE, lysozyme is secreted by epithelioid cells at the "mature" stages of granuloma (**Figure 2**). Thus, this serological marker may be a delayed reaction during the active phase of the disease and not sensitive to track the clinical course of progression or remission. However, it can be used to support diagnosis and follow-up of patients[21].

Serological markers associated with lymphocyte activation

The hallmarks of sarcoidosis are non-caseating granulomas in which activated T cells accumulate at inflammatory sites. Recruitment of peripheral T cells in blood to tissues amplifies inflammatory responses[2]. Sarcoidosis is an "immune paradox," in which the peripheral activity occurs with local exaggerated inflammation[1].

Granuloma formation occurs in 4 stages: initial, accumulation, effector phase, and resolution or fibrosis progression, in which T helper cells are involved in the mechanism of initiation, maintenance, and progressive fibrosis (**Figure 2**[3,22,23]. In the stages of granuloma formation, T cell serological markers may help reveal the systemic inflammatory condition. Here, we discuss the links between clinical progression, dynamics of serological markers, and immunopathogenesis of sarcoidosis.

Interleukin-2 (IL-2) is a cytokine released predominantly by activated $CD4^+$ Th1 cells and has an important role as a growth factor to amplify local immune responses[5]. Activated T cells express IL-2 receptors (IL-2R) consisting of 3 chains on their surfaces, and the α chain is proteolytically cleaved into the surrounding environment in a soluble form[24]. Soluble IL-2R induced by activated human lymphoid cells in vitro was first observed in 1985, and two years later an increase in sIL-2R in the sera of sarcoidosis patients was reported[25,26]. These T cells produced IL-2 and express IL-2α receptor on their surface only after being activated through antigen presentation. The intensity of T cell activation is high during the active disease, but low or absent in chronic sarcoidosis[9]. Therefore, the proteolytic cleavage of the soluble form of α chain from the cell surface into body fluids is a reliable measure of T-cell activation[27]. Several studies have found that sIL-2R is a clinically relevant marker of sarcoidosis to evaluate disease severity and treatment options (**Table 2**).

In 2015, sIL-2R replaced the negative tuberculin test for diagnosis sarcoidosis in Japan (**Table 3**)[28], and a 2017 study found that sIL-2R was more sensitive than ACE or lysozyme for diagnosing patients with cutaneous lesions[6]. About 30% of patients had increased serum sIL-2R levels but normal serum ACE levels, and 19% of patients had elevated serum sIL-2R levels but normal lysozyme levels on their first visit. During follow-up, sIL-2R levels also correlated with clinical progress, but ACE and lysozyme levels were not correlated[6,20,29].

sIL-2R is released by activated T cells, which are recruited at an early phase of granuloma formation and increase during active disease, and sIL-2R is also secreted by activated monocytes, B cells, and alveolar macrophages in sarcoidosis[30]. Therefore, an increased level of serum sIL-2R is a reliable index of ongoing systemic granulomatous inflammation and has higher sensitivity compared to ACE or lysozyme for diagnosing sarcoidosis. In addition, activated T cells target the affected area and proliferate during the initial immune response in the formation of the granuloma, which suggests that sIL-2R is an early marker of active sarcoidosis. Unfortunately, sIL-2R is non-specific because it increases in other conditions[31], but may still be used to identify sarcoidosis in suspected patients with a non-caseating necrosis granuloma in a cutaneous biopsy.

Table 2. Review of literature on sIL-2R in sarcoidosis

Study	Study subjects (number of patients)	Value in diagnosis	Correlation with activity	Correlation with severity	Correlation with ACE	Correlation with CD4 in BALF	Predicting marker	Marker of treatment response
Lawrence *et al.*[25]	Pulmonary sarcoidosis		Suggestive of active pulmonary sarcoidosis					
Kita *et al.*[39]	Sarcoidosis (n = 15)						Predict the presence of the panda or lambda sign	
Petereit *et al.*[40]	Neuro Sarcoidosis (n = 139)	sIL2-R measurement in the CSF may be a valuable tool in the diagnosis and follow-up of patients with neurosarcoidosis.						
Gungor *et al.*[41]	Sarcoidosis (n = 38)			+ extra-pulmonary involvement				
Grutters *et al.*[42]	Pulmonary sarcoidosis (n = 47)		Marker of pulmonary disease activity and extra-pulmonary disease		-	+		-
Ziegenhagen *et al.*[43]	Sarcoidosis patients without indication of treatment at presentation (n = 77)		Patients with high sIL-2R levels, have a significantly greater risk of disease progression		-			
Bargagli *et al.*[44]	Sarcoidosis (n = 60)		Marker of sarcoidosis progression					
Vorselaars *et al.*[45]	Sarcoidosis (n =14)							sIL-2R correlated with lung function improvement during MTX treatment
Miyoshi *et al.*[20]	Pulmonary sarcoidosis (n = 43)			+ pulmonary infiltration				

Table 2. Continued.

Study	Study subjects (number of patients)	Value in diagnosis	Correlation with activity	Correlation with severity	Correlation with ACE	Correlation with CD4 in BALF	Predicting marker	Marker of treatment response
Gundlach *et al.*[46]	Uveitis sarcoidosis (n = 261)	sIL-2R is a more effective marker parameter for sarcoidosis than ACE or chest x-ray in uveitis patients (spec 94%, sens 98%)						
Ina *et al.*[47]	Pulmonary sarcoidosis (n = 28)		+		+			
Keicho *et al.*[48]	Sarcoidosis (n = 70)		+		+			Good marker for follow-up
Ziegenhagen *et al.*[29]	Sarcoidosis (n = 74)		+					
Ina *et al.*[30]	Sarcoidosis (n = 39)		+		+			
Rothkrantz-Kos *et al.*[49]	Sarcoidosis (n = 185)	For pulmonary disease diagnosis: sen 82%, spec 94%, pos predictive value 82%, neg predictive value 94%		Pulmonary disease				Good marker for follow-up
Prasse *et al.*[50]	Sarcoidosis (n = 225)		+	+ extra-pulmonary involvement			+ progressing disease and the need for treatment in patients with acute disease	
Thi Hong Chuyen et al.[6]	Sarcoidosis patients with cutaneous lesions (n = 72)	sIL-2R was more sensitive than both ACE and lysozyme in supporting a diagnosis of sarcoidosis (52.8%) compared to ACE (29%) and lysozyme (26.4%)	+	+ multiple organ involvement, parenchymal infiltration	+		Predicting the presence of multiple organ involvement, pulmonary disease, parenchymal infiltration, and the presence of specific signs in the diagnosis of sarcoidosis.	

Table 3. Diagnostic criteria for sarcoidosis in Japan[28]

(A) Definitive Diagnosis (Clinical and histological evidence group)
More than one clinical sarcoidal lesion with non-caseating necrosis epithelioid cell granulomas, and rule-out other granulomatous disorders and sarcoid reactions.
Note: Careful examination of characteristic laboratory findings for sarcoidosis and systemic involvement is necessary.
(B) Possible Diagnosis (Absence of histological evidence group)
Two of three important organ involvements (lung, eye, and heart) and two of five characteristic laboratory findings are needed for diagnosis.
1. BHL
2. High serum levels of ACE or lysozyme
3. High serum level of sIL-2R
4. Abnormal accumulation in ^{67}Ga scintigraphy or PET-CT
5. An increased number of lymphocytes or a CD4/CD8 ratio more than 3.5 in BALF

Serological markers associated with fibrotic remodeling

T helper type 1 (Th1) responses and Th1-associated cytokines are key players in the pathogenesis of sarcoidosis, but the mechanism of granuloma formation and fibrosis is unknown. Th1 and Th2 cytokine expression patterns in sarcoidosis are important to predict the clinical outcome of an immune response, in which a Th1 response results in an antigen/pathogen clearance and resolution and a Th2 response results in fibrosis remodeling[22].

TARC, also known as chemokine (C-C motif) ligand 17 (CCL17), is a crucial chemokine for the amplification of Th2 responses, which occur by recruiting CCR4-expressing $CD4^+$ T cells.[32-35] Increased serum levels of TARC are implicated in non-specific compensate reactions in the Th1 response in sarcoidosis[36]. Serum TARC levels are elevated in 78% of sarcoidosis patients and expressed by monocyte-macrophage lineage cells within granulomas. In addition, an imbalance between Th1 and Th2 results in a Th2 response that underlies prolonged TARC overproduction. Therefore, TARC is actively involved in the pathogenesis of sarcoidosis. One third of patients with sarcoidosis may have a chronic disease with a high risk of fibrosis. Patients with increased serum TARC levels have a significantly increased incidence of pulmonary infiltration. In the clinical practice, TARC may be good predictive marker for pulmonary fibrotic progression in chronic sarcoidosis patients[37]. Understanding the role of TARC in promoting and maintaining local and systemic inflammatory reactions is important because it may lead to targeted treatments to prevent fibrosis events.

Conclusion

Based on the progression of sarcoidal granuloma, sIL-2R is a more useful marker than other indicators in an early and active condition, and "traditional" indices, such as ACE and lysozyme, are less sensitive for the diagnosis and management of sarcoidosis. TARC is a promising marker, but further investigation on Th2 activity in sarcoidosis and fibrosis mechanisms is needed. Understanding the link between clinical observations and serological markers in sarcoidosis may aid clinicians treating this challenging disease.

Conflict of interest

The authors declare no potential conflicts of interest.

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REVIEW

Exosome-mediated immune regulation and its clinical application

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ABSTRACT

Immune system is a precise mechanism for maintenance of homeostasis by lymphocyte-mediated elimination of extracellular and intercellular pathogens, and abnormal cells in cytokine-, chemokine-, antibody-, and cytotoxic granule-dependent manners. Extracellular vesicles, *e.g*. exosomes, released from multivesicular endosome in immune cells have been known to be a part of the immune system. Exosomes released by antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs) regulate natural killer (NK) cells, $CD8^+$ T cells (Cytotoxic T lymphocytes [CTLs]), and $CD4^+$ T cells (Th cells) including Th1, Th2, and regulatory T (Treg) cells. In the anti-tumor immune system, NK cells and CTLs are mainly involved in the elimination of tumor cells by direct interaction. Recently, we clarified that tumor-infiltrating $CD8^+$ T cells prevent tumor invasion and metastasis by exosome-mediated destruction of tumor stroma consist of mesenchymal stem cells (MSCs) and cancer-associated fibroblasts (CAFs). In this review article, we describe the role of exosomes in controlling immune system and its clinical application.

Keywords: *$CD8^+$ T cell; exosome; extracellular vesicle; mesenchymal cell; tumor metastasis*

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Conventional immune system

NK cells and macrophages are representative innate immune cells that act as a central player at an early stage of pathological conditions such as infection and cancer. In addition, extrathymically differentiated T cells expressing γδ T cell receptor (TCR), and NK T cells expressing NK makers and invariant αβ TCR to α-galactosylceramide (αGalCer) in the context of CD1d are also classified as innate immune cells. On the other hand, intrathymic-differentiated T cells bore αβ TCR can expand vigorously by priming with specific antigen peptide/major histocompatibility complex (MHC) on dendritic cells (DCs), exhibit effector/memory phenotype, and participate in the elimination of abnormal cells, so termed as acquired immune cells[1]. Acquired immunity is composed by $CD4^+$ helper T (Th1 and Th2) cells, $CD4^+$ $Foxp3^+$ regulatory T (Treg) cells, and $CD8^+$ cytotoxic T lymphocytes (CTLs). Interferon (IFN)-γ-expressing Th1 cells support CTL induction in antigenic stimulation by DCs. Interleukin (IL)-4 secreting Th2 cells promote B cell differentiation into antibody-producing plasma cells and tumorigenesis of epithelial cells, and inhibit inflammation. $CD4^+$ $Foxp3^+$ Treg cells suppress antigen-specific T and B cell responses. In addition to Treg cells, myeloid-derived suppressor cells (MDSCs) strongly reduce T and NK cell activites and promote tumor infiltration of Treg cells[2]. Chemokines from macrophages and DCs attract innate immune cells and acquired immune cells in tumor lesion. It has been gradually clarified that exosomes released from these immunocompetent cells play a part of complicated immune responses[3].

Exosomes from T cells and NK cells

T cells strongly release exosomes with activation[4]. Treg cell exosomes have been studied to some extent, all of which are reports regarding immunosuppressive function. CD73 on Treg cells converts extracellular ATP to immunosuppressive adenosine (ADO), and inhibits T cell and NK cell activities. Treg cell exosomes also express CD73 and seem to participate in the immunosuppression[5,6]. Treg cell exosomes inhibit strongly Th1 cell

activity in an exosomal micro (mi) RNA-denpendent manner[7]. Transforming growth factor (TGF)-β and suppressive miRNAs in breast milk exosomes are retained relatively stable against temperature, pH, and freeze-thaw, and they maintain Treg cells, and prevent the onset of modern diseases such as atopic dermatitis by reduction of IgE production of B cells[8,9]. Suppressive role of Treg cell exosomes may be applicable in tolerance induction during organ transplantation[10].

$CD8^+$ T cells proliferate predominantly when spleen cells of mouse and human peripheral blood lymphocytes are cultured by stimulation with both CD3 and CD28. It has been reported that exosomes from primary culture of T cells preferential activate naive $CD8^+$ T cells, but not naive $CD4^+$ T cells[11]. $CD8^+$ T cells express FasL capable of apoptosis of Fas^+ tumor cells. However, FasL on $CD8^+$ T cell exosomes seem to promote invasion and metastasis of tumor cells, but not tumor cell killing, by matrix metalloproteinase (MMP)-9-medaited degradation of extracellular matrix proteins via Fas/FasL signaling pathway[12]. Conversely, since FasL was not found on $CD8^+$ T cell exosomes in our study, we examined the action of murine $CD8^+$ T cell exosomes in tumors in comparison with exosomes from other lymphocyte populations, tumor cells, or human T cells. Surprisingly, $CD8^+$ T cell exosomes exhibit cytotoxicity against tumor stromal cells such as MSCs and CAFs, but not tumor cells. In addition, $CD8^+$ T cell exosome-mediated destruction of mesenchymal stroma associated with the reduction of tumor invasion and metastasis (Figure 1)[13].

Studies of NK cell exosomes have not progressed because of lack of explosive growth potential compared to T cells and difficulty of cultivation in a single population. NK cell exosomes express FasL, and seem to be able to induce apoptosis of Fas+ tumor cells. It has been reported that cytotoxic substances, perforin and granzyme B, are abundantly shown in the lumen of NK cell exosomes[14].

Relationship among B cells, macrophage, and exosomes

B cells and macrophages have been known as antigen-presenting cells. Major histocompatibility complex (MHC) class II molecules express on exosome membrane released from both cells[15]. B cell exosomes entering lymph node are rapidly taken up and decomposed by subcapsular macrophages. Interaction CD169 (Siglec-1) expressed on subcapsular macrophages with sialic acid on B cell exosomes is important in this setting[16]. It has been reported that exosomes from chronic B leukemia cells have a potential to differentiate vascular endothelial cells and MSCs into smooth muscle actin+ CAFs.

When exosomes are administered systemically, most of them are engulfed by hepatic macrophages and digested in the lysosome. Scavenger receptors

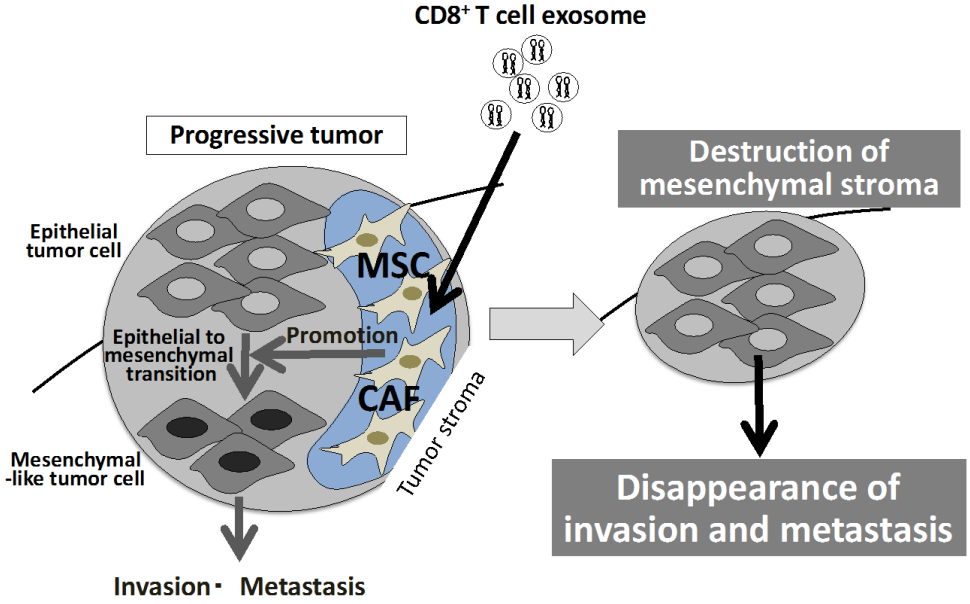

Figure 1. Prevention of tumor progression by CD8+ T cell exosomes. $CD8^+$ T cell exosomes deplete mesenchymal tumor stromal cells such as MSCs and CAFs. This exosome-mediated destruction of tumor stroma associates with reduction of tumor invasion and metastasis.

such as SR-A (Scavenger receptor class A) on hepatic macrophages seem to be a ligand for phosphatidylserine-derived negative surface charge on exosomes[17,18]. In liver metastasis of pancreatic cancer, pancreatic cancer cell-released exosomes participate in the formation of pre-metastasis niche by promotion of TGF-β and fibronectin production of hepatic stellate cells after kupffer cell activation[19], showing close relationship between exosomes and liver macrophages. Exosomes released from tumor-associated macrophages seem to promote differentiation of monocytes into macrophages in an exosomal miRNA-dependent manner[20].

DC exosomes and immune regulation

DCs reside in lymph nodes, skin and mucosal tissues in immature form. By capturing viral antigens or tumor proteins, immature DCs activate with enhanced expression of MHC class II molecules, migrate into draining lymph nodes, and present antigen peptides to specific T cells[21]. Immature DCs release exosomes more vigorously than mature DCs[22,23]. Immature DC exosomes exhibit a potent role in Treg cell activation[23,24], suggesting maintenance of homeostasis by suppressing autoimmune reactions and excessive inflammations. Whereas, mature DCs release exosomes to facilitate tumoricidal immune reactions.

Hence DC exosomes pulsed with tumor antigen peptides can induce cytotoxic T lymphocyte (CTL) responses in B cell dependent manner (unknown mechanism) *in vivo*, clinical application of DC exosomes have already begun (Figure 2)[25–27]. However, DC exosomes seem to have high NK cell activating capacity rather than CTL induction[25,26,28]. DC exosomes pulsed with α-galactosylceramide (α-GalCer) and tumor antigen peptides can activate invariant (i) NK T cells and γδ T cells concomitant with specific CTLs, resulting elicitation a strong antitumor immune responses[29]. MHC class I molecule on the exosome membrane exists as dimer at the end of the lumen region, and may have different conformation from that of the monomer[30]. Furthermore, exosomes derived from DCs from MHC class I knockout mice have T cell inducing and NK cell activating abilities comparable to those of DC exosomes from normal mice[31], suggesting MHC class I-independent mechanism of antigen presentation by DC exosomes (Figure 2).

Tumor cell exosomes and immune regulation

Immune modulatory effect of tumor cell exosomes was most developed. Tumor cell-derived exosomes promote activation and accumulation of Treg cells[32–34]. Likewise, tumor cell exosomes enhance production of prostaglandin E2, IL-6, and TGF-β of MDSCs, resulting formation of

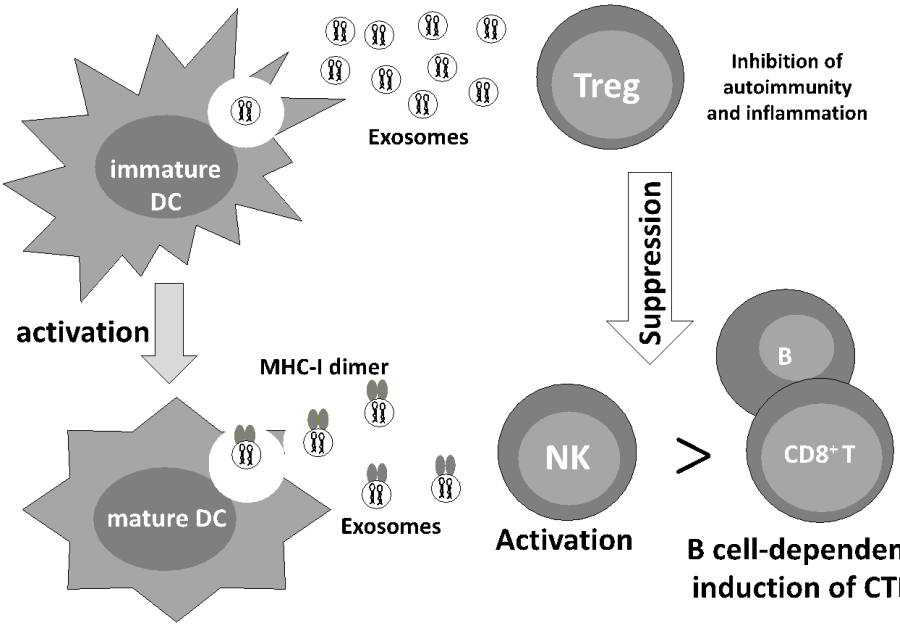

Figure 2. DC exosome-mediated activation of immune cells. Immature DCs and mature DCs release exosomes to induce Treg cells, and NK cells and CTLs, respectively. MHC class I molecules on exosomes from mature DCs express as a dimer.

strong immunosuppressive environment in tumor lesions[35,36]. NK cells, γδ T cells, and a part of CTLs recognize tumor surface MHC class I molecule-like UL16-binding protein (ULBP) [3] and MHC class I polypeptide-related sequence A (MIC-A) by interaction with NKG2D, and can lyse tumor cells. However, tumor cells released ULBP- and MIC-A-bearing exosomes, and block cytotoxicity[37,38]. Interestingly, it has been reported that tumor cell exosome-engulfed DCs produce immune competent exosomes for effective induction of anti-tumor immunity[39,40]. This seems to be related to the Type-I IFN secretion mediated by cGAS (cyclic GMP-AMP synthase)/STING (Stimulation of *IFN* gene) pathway in DCs by exosomal DNAs[41].

Tumor cells are always under hypoxic conditions and temperature stress, and are also exposed to drug stress during treatment of anticancer agents. Under these circumstances, it has been known that tumor cells release exosomes more aggressively than normal condition, and exhibit immune modulatory effects. In low oxigen, tumor cells released TGF-β-bearing exosomes, and promote and inhibit Treg cell activity and NK cell cytotoxicity, respectively[42]. Conversely, tumor cell exosomes released by high temperature stress or anticancer drug stress embed HSP (Heat shock protein)-70 and CCL (CC chemokine) -2, -4, -5, and -20 capable of promoting migration and activation of T cells, NK cells, and DCs[43,44].

Concluding remarks

In immune system, exosomes seem to inherit the function of parent cells, implying exosomes from $CD8^+$ T cells, macrophages, and B cells for treatment of tumors in addition to the already used DC exosomes. However, proteins expressed or embedded in exosomes may exhibit different function from those in the parent cells, as shown in MHC class I dimer on exosome membrane and MHC class I-independent activation of NK and CTLs by DC exosomes. Solving various issues including molecular structure should be important to elucidate the biological significance and clinical treatment of immune cell-derived exosomes.

Conflict of interest

The author declares no potential conflict of interest with respect to the research, authorship, and/or publication of his article.

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SHORT REPORT

Evaluation of the safety and usefulness of *citrus jabara* fruit peel powder cream for patients with atopic dermatitis

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ABSTRACT

Citrus jabara (CJ) is a rare citrus that originally grew wild only in the southern area of the Kii peninsula in Japan. In the relationship between CJ and atopic dermatitis (AD), improvement of AD by oral intake of CJ fruit juice was reported in AD model mice. Our previous study also showed anti-inflammatory potentials of CJ fruit peels *in vitro*. In this study, the applicability of CJ fruit peel powder (CJ powder) for topical application in patients with AD was investigated. After confirming both the safety of CJ powder in preclinical studies and the safety of 5% CJ powder cream in healthy volunteers, the safety and usefulness of 5% CJ powder cream were evaluated in 20 patients with AD. Evaluation of 5% CJ powder cream in patients with AD for 4 weeks showed improvement in the mean severity score of the affected area (from 3.0 to 2.0, p=0.001 by Student's t-test), improvement in skin lesions (11 of 20 participants), usefulness (16 of 20 participants), and safety (16 of 20 participants). Although aggravation of symptoms on application areas were observed on 4 participants, their aggravation were systemic, resulting from causes other than tested cream. These results suggested that 5% CJ powder cream is useful and safe for patients with AD.

Keywords: *Citrus jabara; Atopic Dermatitis; External Cream; Narirutin; Safety; Usefulness*

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Introduction

Citrus jabara (CJ) is a rare citrus that originally grew wild only in the southern area of the Kii peninsula in Japan. Our previous study showed that almost all of flavonoids in CJ fruit peels was narirutin, and CJ fruit peels have anti-inflammatory potentials to inhibit productions of nitric oxide (NO) and interleukin-6 (IL-6), and to inhibit enzymatic activities of soluble epoxide hydrolase (sEH) and hyaluronidase[1]. The inhibitory effect of oral administration of CJ fruit juice was observed on the development of atopic dermatitis (AD) -like skin lesions in NC/Nga mice[2]. Narirutin, which is considered as a main functional component in CJ fruit, is present in peel much more than in juice. These findings expected that CJ fruit peels are effective in improving AD. In this study, we investigated the applicability of CJ fruit peel powder (CJ powder) for topical application in patients with AD.

Materials and Methods

Preparation of CJ powder

The CJ powder was prepared in accordance with Japanese Patent No. 5,323,127 as follows[3]. Briefly, CJ fruits, harvested on early November in 2012, were wholly pressed to remove juice. CJ fruit peels, residue of squeezed CJ fruits, were firstly freeze-dried to remove monoterpenes, next pasteurized, and then powdered to 200 mesh pass particles.

Five percent CJ powder cream

The 5% CJ cream was made by mixing 5% by weight of CJ powder into the base cream consisting of 1,3-butylene glycol, mineral oil, cetyl ethylhexanoate, glycerin, polyacrylamide, dipotassium glycyrrhizate, tocopherol, polysorbate 60, hydrogenated polyisobutene, laureth-7, phenoxyethanol, methylparaben, and purified water. All reagents except for CJ powder were registered in Japanese Standards of Quasi-drug Ingredients (2016).

Component analysis of CJ powder

Amounts of (R)-limonene oxide and (R)-(-)-carvone were quantified by GC-MS. Quantitative analysis was performed with purchased standard samples (Wako Purechemical, Osaka, Japan). The minimum limits of determination of each samples were follows: (R)-limonene oxide, 0.5 μg/ g; (R)-(-)-carvone, 0.3 μg/ g, respectively.

Amount of narirutin was quantified by HPLC with standard sample (Wako Purechemical, Osaka, Japan). Briefly, Chromatographic separation was performed on a COSMOSIL 5C18-AR-II column (φ 4.6 x 250 mm) with a following condition: temperature, 40 ˚C; mobile phase, 20% acetonitrile containing 0.8% acetic acid; flow rate, 1.0 mL/ min. Quantitative analysis was performed with purified standard sample of narirutin. Peaks were detected at 280 nm. The minimum limit of determination was 0.25 μg/ ml.

Preclinical safety evaluation of CJ powder

Following 9 safety tests of CJ powder were carried out in Biosafety Research Center (Sizuoka, Japan) according to OECD Principles of Good Laboratory Practice (GLP) (ENV/MC/CHEM(98)17); acute oral toxicity test, acute skin irritation test, acute eye irritation test, local lymph node assay (LLNA) , Ames test, cumulative skin irritation test, photosensitization test, phototoxicity test, and chromosome aberration test.

Safety evaluation of CJ powder and 5% CJ powder cream in healthy volunteers

Patch tests of CJ powder and 5% CJ powder cream were carried out in Nikoderm Research Inc (Osaka, Japan). Each patch test was carried out with 40 healthy volunteers for 24 hr. Skin sensitization test with 71 healthy volunteers for 6 weeks, and skin phototoxicity test with 32 healthy volunteers for 9 days were carried out in Allergisa Pesquisa Dermato-Cosmética Ltda (Campinas, Brasil).

Clinical evaluation of usefulness and skin safety of CJ powder cream in patients with AD

Participants

The participants were patients with mild to moderate AD who visited Wakayama Medical University Hospital Department of Dermatology between January 2016 and March 2017. The patient met the diagnostic criteria for atopic dermatitis[4,5].The participant, who had been using topical steroid, stopped using steroid during a clinical evaluation period.

CJ powder cream application

The participants applied 1 FTU of 5% CJ powder cream twice a day (morning and evening, or after bathing) on single-site forearm flexor with rash at home for 4 weeks.

Observation and assessments

This was single arm, non-randomized, before-after trial. The usefulness and safety of 5% CJ powder cream were evaluated on interview, visual inspection, and palpation of skin findings by doctor in charge every week. For the severity diagnosis, if erythema, papule, itching, dryness, and lichenization were observed as symptoms of the skin lesions part, 1 point was added to each, and the severity was judged as a minimum of 0 point and a maximum of 5 point. The usefulness of 5% CJ powder cream was comprehensively evaluated by two medical doctors in charge after 4 weeks of use on a five-point scale as follows; extremely useful, useful, slightly useful, not useful, and useless. The safety of 5% CJ powder cream was evaluated by doctor in charge after 4 weeks of use on a four-point scale as follows; safe, almost safe, insufficiently safe, and not safe. Patients and medical doctors didn't know if it's a placebo or an active drug.

Statistical analysis

Student's *t*-test was used to analyze the severity diagnosis between before and after use of 5% CJ powder cream.

Ethics Statement

The ethical committee of Wakayama Medical

University approved this study (Approval number 1716). All study participants were provided informed consent.

Results

Component analysis of CJ powder

The contents of (R)-limonene oxide and (R)-(-)-carvone, inducers of contact dermatitis, in CJ powder were below the lower limit of quantification. The content of narirutin, active ingredient, in CJ powder was 64.4 mg/ g.

Safety evaluation of CJ powder

Safety of CJ powder was assessed by preclinical study and with healthy volunteers. CJ powder was recognized as safe in all evaluation items (**Tables 1** and **2**).

Table 1. Preclinical safety evaluation of CJ powder

Evaluation item	Evaluation result
Acute oral toxicity test	safe
Acute skin irritation test	safe
Acute eye irritation test	safe
LLNA test	safe
Ames test	safe
Cumulative skin irritation test	safe
Photosensitization test	safe
Phototoxicity test	safe
Chromosome aberration test	safe

Safety evaluation of CJ powder cream in healthy volunteers

Safety of 5% CJ powder cream was sessed by patch test, skin sensitization test, and skin phototoxicity test in healthy volunteers. The safety of 5% CJ powder cream was found in all evaluation items (Table 2).

Table 2. Safety evaluation of CJ powder and 5% CJ powder cream in healthy volunteers

Evaluation item	Evaluation result
Patch test of CJ powder	safe
Patch test of 5% CJ powder cream	safe
Skin sensitization test of 5% CJ powder cream	safe
Skin phototoxicity test of 5% CJ powder cream	safe

Background of participants in clinical evaluation

The participants comprised 10 men and 10 women whose mean age was 37.1 ± 10.1 (22-59 years old).

Clinical evaluation of usefulness and skin safety of 5% CJ powder cream in patients with AD

The severity score before and after 5% CJ powder cream treatment, usefulness of 5% CJ powder cream, and the safety of 5% CJ powder cream were assessed in 20 patients with mild to moderate AD. Among them, 2 participants were dropped out before the end of the study.

The mean severity score was 3.0 at baseline and 2.0 at Week 4 (**Figure 1A**). Application of 5% CJ powder cream significantly reduced severity score (p=0.001 by Student's t test). The 5% CJ powder cream was evaluated as useful (extremely useful, useful, and slight useful) in 16 participants (**Figure 1B**). Improvement in skin lesions was observed in 11 participants. The safety of 5% CJ powder cream was observed in 16 of 20 participants (**Figure 1C**). Although aggravation of symptoms on application areas were observed on 4 participants, their aggravation were systemic, resulting from following factors; such as, heat and humidity in summer, overwork because of continuous night shift, and excessive alcohol drinking. Thus, adverse events associated with tested cream were not observed.

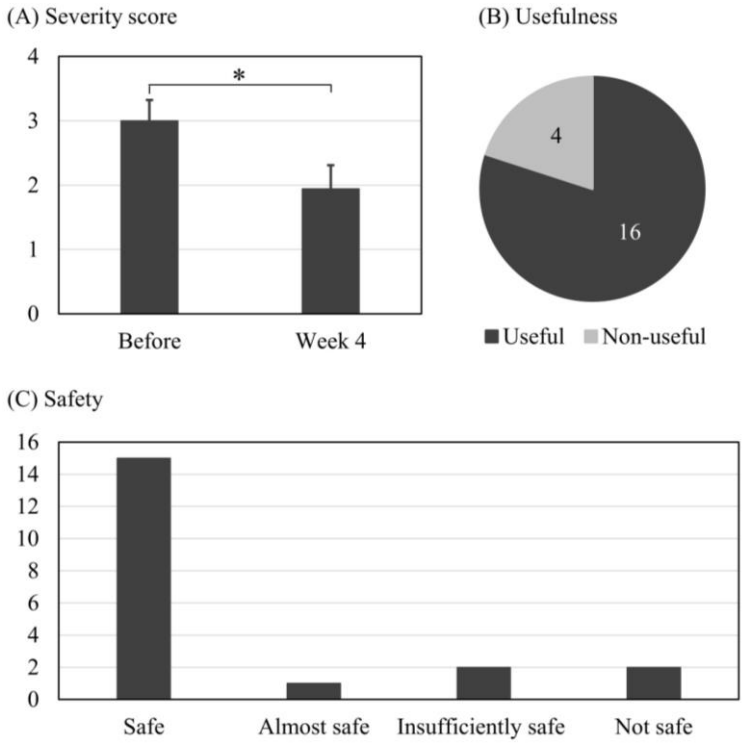

Figure 1. Clinical evaluation of 5% CJ powder cream in patient with AD. (A) Changes in severity scores, (B) usefulness, and (C) safety of 5% CJ powder cream in patients with mild to moderate AD were evaluated for 4 weeks. The participants applied 1 FTU of 5% powder cream twice a day (morning and evening, or after bathing) on single-site forearm flexor with rash at home for 4 weeks. For changes in severity score, Student's *t*-test was used to compare two groups, and significant difference was found (* P=0.001). Bars indicate standard error.

Discussion

Treatments for AD are diverse. We have been studying the usefulness of lactic acid bacteria[5], borage oil[6] and others. Based on these results, we planned this research.

In this study, we investigated the applicability of CJ powder for topical application in patients with AD. Prior to clinical study, the safety of CJ peel powder was examined, because there were no studies on CJ peels as a topical application. When using citrus peels, the following two compounds should be noted: monoterpene oxides and furanocoumarins. Monoterpenes, such as limonene, are aromatic components of citrus, but these oxides can cause contact dermatitis[7]. These oxides are remarkably increased during heating in the presence of oxygen[8]. We solved this problem by removing monoterpenes by the freeze-drying method prior to heat drying[3]. Both (R)-limonene oxide and (R)-(-)-carvone, the oxides of limonene, which are inducers of contact dermatitis, were below the limit of quantification in CJ powder. Furanocoumarins are phototoxic compounds known to be present rich in grapefruit[9]. We did not find any furanocoumarins in CJ peels, although we have comprehensively isolated and identified the compounds in them. Not all citrus fruits contain furanocoumarins, some contain more of them, such as grapefruit and bitter orange, or even none at all, such as *Citrus iyo*[10]. Therefore, it is not surprising that furanocoumarins could not be found in the CJ peel. The results of preclinical studies (Table 1) and patch tests in healthy volunteers (Table 2) also confirmed the safety of CJ peel.

The 5% CJ powder cream was first evaluated for safety in healthy volunteers, and then for usefulness and skin safety in patients with AD. The 5% CJ powder cream was found to be safe on the healthy skin, as it was negative in patch tests, skin sensitization tests, and skin phototoxicity tests in healthy volunteers (Table 2). Evaluation of 5% CJ powder cream in patients with AD for 4 weeks showed improvement in mean severity score of the affected area (from 3.0 to 2.0), improvement of eruption (11 of 20 participants), usefulness (16 of 20 participants), and safety (16 of 20 participants) (Figure 1). During this study, the use of topical steroids was stopped for all participants, thus, it was feared that participants, who had been using topical steroids, were in danger of worsening their symptoms due to the rebound phenomenon, but nonethe-

less, safety and usefulness of 5% CJ powder cream were found in 80% of the participants. Although aggravation of symptoms on application areas were observed on 4 participants, their aggravation were systemic, resulting from causes other than tested cream. These results suggested that 5% CJ powder cream is useful and safe for patients with AD.

Previous studies on CJ and its components have shown that CJ is effective in treating allergic diseases such as AD[2], asthma[11] and hay fever[12], and its functional component is considered to be narirutin. The results of this study are consistent with the results of these previous studies. However, there is a major difference between the previous studies and the present study in the way of ingestion of CJ. That is, all the previous studies were taken orally and the present study was applied to the skin. Comparing the amount of narirutin in the present study with previous studies, it is estimated to be 3.2 mg/day in the present study, 10-20 mg kg^{-1}/day in the AD model mice[2], 10 mg kg^{-1}/day in the asthma model mice[11], and 11 mg/day in human hay fever[12]. Comparing the methods of narirutin administration, application to the skin is considered to be as effective as or more effective than oral administration. In oral intake, narirutin (molecular weight: 580.5) is aglyconized by intestinal bacteria and absorbed as naringenin (molecular weight: 272.3). In *in vitro* experiments, naringenin is significantly stronger than narirutin in terms of anti-inflammatory effects[1]. Therefore, although speculative, this study suggests that the deglycosylation of narirutin may have occurred by the skin flora present in the affected area of the skin and/or enzymes released by immune cells, such as granulocytes and macrophages.

Conclusion

CJ powder cream is considered as useful and safe for patients with AD.

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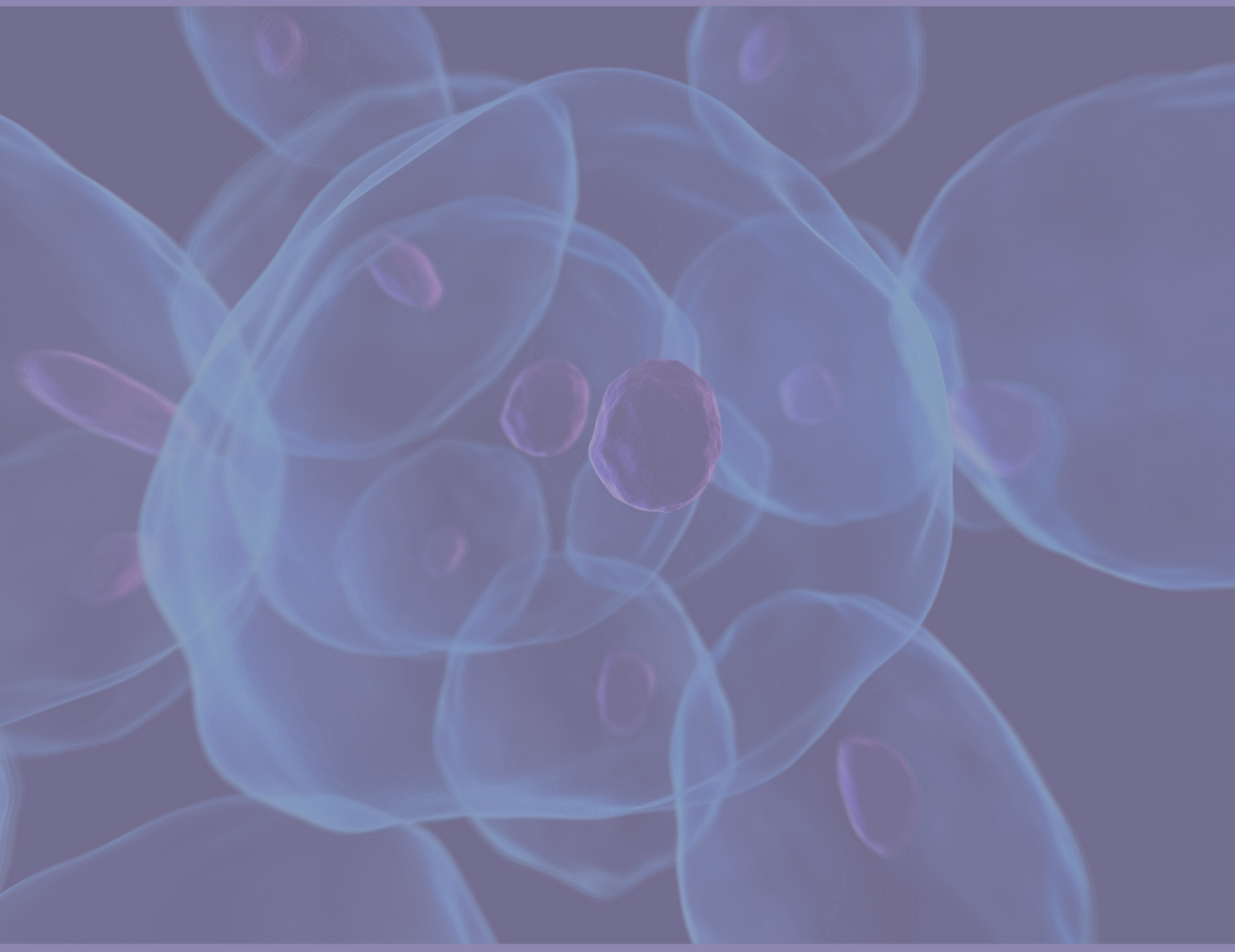

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