Original Article

Coronary Vasculitis Induced in Mice by Cell Wall Mannoprotein Fractions of Clinically Isolated Candida Species

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ABSTRACT

Kawasaki disease (KD) is an inflammatory disease that was identified by Professor Tomisaku Kawasaki in 1961. Candida albicans-derived substances (CADS) such as the hot water extract of C. albicans and Candida water-soluble fractions (CAWS) induce coronary vasculitis similar to KD in mice. An increasing proportion of deep-seated candidiasis cases are caused by non-albicans Candida and are often resistant to antifungal drugs. We herein investigated whether the mannoprotein fractions (MN fractions) of clinically isolated Candida species induce vasculitis in mice. We prepared MN fractions from 26 strains of Candida species by conventional hot water extraction and compared vasculitis in DBA/2 mice. The results obtained revealed that the induction of vasculitis and resulting heart failure were significantly dependent on the species; namely, death rates on day 200 were as follows: Candida krusei (100%), Candida albicans (84%), Candida dubliniensis (47%), Candida parapsilosis (44%), Candida glabrata (32%), Candida guilliermondii (20%), and Candida tropicalis (20%). Even for C. albicans, some strains did not induce vasculitis. The present results suggest that MN-induced vasculitis is strongly dependent on the species and strains of Candida, and also that the MN fractions of some non-albicans Candida induce similar toxicity to those of C. albicans.

Key words: Candida species, dectin-2, innate immunity, mannoprotein, vasculitis

Introduction

Kawasaki disease (KD) is an inflammatory disease that typically develops in children, particularly those younger than 4 years old, and was identified by Professor Tomisaku Kawasaki in 1961.1-3) The number of KD patients has continued to increase annually. Although intravenous immunoglobulin (IVIG) is the standard treatment, some patients develop coronary vasculitis and are at risk of developing cardiovascular disease later in life.4-7) Despite extensive research, the etiology of KD remains unclear. Recent advances have increased the accuracy of diagnoses and resulted in the development of therapeutic strategies for better acute as well as long-term outcomes.5-7,8)

The induction of coronary vasculitis in mice was originally demonstrated by Murata in 1978 by injecting Candida albicans-derived substances (CADS) obtained from a KD patient, and Naoe and Takahashi repeatedly investigated CADS-induced coronary vasculitis from the viewpoint of pathology.3) We demonstrated that the C. albicans water-soluble fraction (CAWS) induced a similar pathology in mice.10) We and others reported that CADS and CAWS induced aortitis and coronary vasculitis in mice, which resembled those in KD patients; therefore, this is a suitable animal model for examining the vasculitis of KD. Previous studies demonstrated the contribution of various factors to the induction and prevention of CAWS-vasculitis. We demonstrated that the vasculitis activity of CAWS negatively correlated with the presence of β-mannose residues.11,12) We also confirmed that coronary vasculitis was induced by the hot water extract (HWE) of the standard strains of Candida.

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Received: 18, March 2020, Accepted: 23, June 2020
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albicans, Candida metapsilosis, and Candida krusei. Chemical and NMR spectral analyses strongly suggested that the major polysaccharide component of HWE is the cell wall mannans, thus the vasculitis activity might be strongly related to the presence and the structure of mannan moiety.

CAWS-vasculitis, particularly in DBA/2 mice, is severe and ultimately results in death due to cardiac hypertrophy. The progression of CAWS-vasculitis in DBA/2 mice has been classified into the following three stages: 1) development of vasculitis, 2) complex myocardial remodeling with hypertrophy, and 3) fatal severe left ventricular dysfunction and sudden death. The series of changes associated with CAWS-vasculitis are characterized as chronic inflammatory diseases, possibly due to irreversible reactions.

Candidiasis is a common fungal infection caused by yeasts belonging to the genus Candida and includes mucosal candidiasis, cutaneous candidiasis, and systemic candidiasis. The most prevalent species is Candida albicans. Candida yeasts are generally present in healthy humans as components of the microbiome. The pathogenic factors of Candida species have been extensively analyzed, such as the production of adhesive factors and hydrolases, yeast-to-hypha transition, and biofilm formation. In these studies, the cell wall mannans were often extracted using hot water extraction. The resulting crude mannan fractions (MN fractions) from Candida albicans were often extracted using hot water extraction of the clinically isolated strain of Candida albicans NBRC1385 and have been extensively examined. Manan is a known as a species- and strain-dependent antigen. Receptors for mannan, such as the mannose receptor, mannan-binding protein, and dectin-2, have been extensively examined. Dectin-2 is a type II transmembrane C-type lectin receptor (CLR) that is the most abundantly expressed on tissue macrophages and inflammatory monocytes. Knockout mice for dectin-2, the innate immune receptor for mannan, were recently shown to be resistant to CAWS-vasculitis.

Although C. albicans is the most prevalent species involved in mucocutaneous and disseminated infections, the incidence of candidiasis due to non-albicans Candida species is increasing, with C. glabrata, Candida tropicalis, Candida parapsilosis, and C. krusei being the main isolates. These species are inherently resistant or acquire resistance, or both, to commonly used antifungal drugs, such as fluconazole, and, thus, the identification of species of Candida is important for selecting therapeutic strategies. Since the mannoprotein structures of C. albicans and non-albicans Candida differ, their toxicological and biological activities may also vary.

Since the goal of animal studies is to develop better therapeutic strategies for KD patients, we want to compare the vasculitis activities of clinically isolated Candida species. The purpose of this study is to clarify the species as well as strain dependency of vasculitis induction in Candida. Historically, the induction of coronary vasculitis in mice was demonstrated by injecting the crude cell wall MN fraction (CADS) prepared using hot water extraction of the clinically isolated strain of C. albicans. It is of note that the cell wall mannoprotein structure is significantly dependent on the species, strains, as well as culture conditions; thus, it would be interesting to know the structural factors responsible for vasculitis activity.

To investigate these topics, we attempted to prepare the extracellular MN fractions (CAWS-like molecules) from several Candida strains; however, the yield of the extracellular fractions significantly differed in a strain-dependent manner (data not shown). In addition, we have prepared the cell wall MN fractions from C. albicans NBRC1385 and have demonstrated the similarities in vasculitis and anaphylactoid shock induced by these fractions and by CAWS in vivo. In the present study, we prepared MN fractions from clinically isolated Candida species and examined coronary vasculitis induced by these fractions. Using in vivo animal model for screening required sufficient quantities of MN fractions, thus, we used the hot-water-extracted MN fractions.
Materials and methods

Materials

Tween 20 was purchased from Wako Pure Chemical Co. (Osaka, Japan). Anti-human IgG (Whole Molecule) - peroxidase conjugate and bovine serum albumin (BSA) were from Sigma-Aldrich Co., LLC. (St. Louis, MO). TMB Microwell Peroxidase was from Kirkegaard & Perry Laboratories, Inc. (KPL) (Gaithersburg, Maryland).

Collection of Candida species

Clinically isolated Candida species (n = 52) were obtained from the stock culture collection of the Medical Laboratory, Kyorin University Hospital. The microbes were handled in accordance with the hospital guidelines. A standard strain, C. albicans NBRC 1385, was obtained from the National Institute of Technology and Evaluation Biological Resource Center (NBRC).

Preparation of crude mannan fraction (MN fraction) by hot water extraction of Candida species

The completely synthetic medium, C-limiting medium, contained (per liter): sucrose 10 g, (NH₄)₂SO₄ 2 g, KH₂PO₄ 2 g, CaCl₂ 2H₂O 0.05 g, MgSO₄ 7H₂O 0.05 g, ZnSO₄ 7H₂O 1 mg, CuSO₄ 5H₂O 1 mg, FeSO₄ 7H₂O 0.01 g, and biotin 25 mg (final pH, 5.2). The natural medium, YPD medium, contained (per liter): yeast extract 10 g, Bacto peptone 20 g, and D-glucose 20 g (final pH, 5.2). C-limiting medium and YPD medium (200 mL) were prepared in 500-mL Sakaguchi flasks and cultured at 27ºC (CL-medium) or 37ºC (YPD medium) with reciprocal shaking for 48 hours. Following the culture, an equal volume of ethanol was added to kill yeasts. Cells were then extensively washed with distilled water. The resulting cells were suspended in 50 ml of distilled water and then autoclaved at 128ºC for 4 h. This suspension was allowed to cool to room temperature (RT). After centrifugation, the supernatant was carefully collected and dried with ethanol and acetone. In the present study, the hot water extract (HWE) was used as the crude mannan fraction (MN fraction).

Preparation of CAWS

CAWS was prepared from C. albicans strain NBRC1385 in accordance with conventional methods. The procedure used was as follows: 5 L of medium (C-limiting medium) was added to a glass incubator and cultured at 27°C for 2 days with air supplied at a rate of 5 L/min and rotation at 400 rpm. Following the culture, an equal volume of ethanol was added, and after the mixture was allowed to stand overnight, the precipitate was collected. The precipitate was suspended in 250 mL of distilled water, and the solubilized fraction was collected. Ethanol was added to the solubilized fraction, and the mixture was left to stand overnight. The precipitate was collected and dried with acetone to obtain CAWS.

Physicochemical analysis

Total carbohydrate concentrations were measured using the phenol-sulfuric acid method with D-glucose as the standard. Total protein concentrations were assessed using the BCA Protein Assay Reagent Kit (PIERCE Biotechnology) with BSA as the standard.

Microplate assays for detecting mannoproteins

Ninety-six-well half-area microplates (Greiner Bio-one) were coated with CAWS, mannan, or MN fractions (25 µg/ml) dissolved in 0.1 M phosphate buffer, and incubated at 4°C for 24 h. After blocking by 1.0% BSA/PBST (with Ca/Mg), serial dilutions of recombinant h-dectin-2 conjugated with the human Ig-Fc fragment (Sino Biological) were added (maximum concentration of 1.0 µg/mL). After the incubation, anti-human IgG-POX was added to detect recombinant dectin-2. After extensive washing, plate-bound dectin-2 was detected by TMB reagent.

Mice

Male DBA/2 and ICR mice were purchased from Japan SLC. Mice were maintained under specific pathogen-free (SPF) conditions at 23 ± 1ºC with a constant humidity of 55 ± 5% under a 12-h light/dark cycle, and had free access to food (CE-2) and tap water in accordance with the Guidelines for Experimental Animal Care issued by the Prime Minister’s Office of Japan. Mice were used in the present study at 5 weeks of age. All animal experiments followed the guidelines for laboratory animal experiments by Tokyo University of Pharmacy and Life Sciences (TUPLS), and each of the experimental protocols was approved by the Committee of Laboratory Animal Experiments in TUPLS (P15-42).

Assay for the anaphylactoid reaction

The indicated doses of the MN fractions and CAWS were dissolved in PBS and intravenously (i.v.) administered to ICR mice. The incidence and severity of rapid anaphylactoid shock were assessed within 30 min.

Administration schedule for the induction of coronary vasculitis

After a 1-week acclimation period, the MN fractions and CAWS dissolved in PBS were administered intraperitoneally (i.p.) for 5 consecutive days to each mouse in order to measure survival or vasculitis. To assess vasculitis, we fixed the hearts of animals with 10% neutral formalin and prepared them in paraffin blocks. Tissue sections were stained with hematoxylin & eosin (HE).
Statistical analysis
Results are expressed as means and standard deviation (SD). The significance of differences between means was measured using the Student’s t-test. Survival rates were analyzed using the Kaplan-Meier method, and the significance of differences was assessed using the log-rank test.

Results
Setting the experimental design for studying vasculitis induced by clinically isolated Candida strains
Two clinically isolated strains, C. albicans KYUP#3 and C. krusei KYUP#35, were used to set up the experimental protocol to analyze vasculitis-inducing activity. These strains were cultured for 2 days using natural (YPD medium) and chemically defined (C-limiting medium) media, and the crude cell wall mannoprotein fractions (MN fraction) were prepared using hot water extraction, in accordance with the method used for standard strains 13, 15, 16).

The resulting MN fractions were abbreviated as CAC3 (C. albicans C-limit #3), CAY3 (C. albicans YPD#3), CKC35, and CKY35, respectively. Yields were 350 mg (400 mL culture), 336 mg, 135 mg, and 302 mg, respectively. In preliminary experiments, all MN fractions strongly reacted with Concanavalin A and dectin-2, suggesting the presence of a significant amount of the α-mannan structure (data not shown). Since standard strains caused severe acute shock and severe lethal vasculitis, these activities induced by MN fractions were tested using standard experimental protocols. Acute toxicity was assessed in ICR mice i.v. administered with MN fractions at a dose of 400 µg/mouse. The numbers of deaths/total mice caused by CAC3, CAY3, CKC35, and CKY35 were 0/3, 2/3, 3/3, and 2/3, respectively. Lethal vasculitis in DBA/2 mice caused by these MN fractions was then examined. CAWS was used as a positive control. MN fractions (1 mg each) were i.p. administered to DBA/2 mice (7 mice each) for 5 consecutive days. Three weeks after the final administration, some mice (2 mice each) were sacrificed to prepare tissue sections of the aortic region (Fig. 1). Mice administered with CAWS, CAY3, CKC35, and CKY35 showed significant vasculitis, whereas those administered with CAC3 did not. Furthermore, the heart weights for CAWS, CKC35, and CKY35 increased, possibly due to heart failure. The remaining mice (5 mice each) were housed for another 18 weeks. Fig. 2 shows the survival days and body weights of these mice. As shown in Fig. 2, all mice administered with CAWS, CAY3, CKC35, and CKY35 died, whereas those administered with CAC3 did not. The kinetics of body weights was similar to the induction of vasculitis, namely, body weight decreased with heart failure. CAY3, CKC35, and CKY35 all induced acute toxicity and lethal vasculitis, whereas CAC3 did not. These results strongly

| HE staining of aorta |
|----------------------|
| Control* | CAWS | CAC3 | CAY3 | CKC35 | CKY35 |
| Control* | CAWS | CAC3 | CAY3 | CKC35 | CKY35 |
| HW**   | 265 mg | 185 mg | 189 mg | 219 mg | 219 mg |
| BW***  | 21.7 g   | 21.7 g   | 21.0 g   | 19.0 g   | 20.4 g   |

*HE section of control mouse was prepared in a separate experiment.
**Mean heart weight at 3 weeks.
***Mean body weight at 3 weeks.

Fig. 1. Vasculitis induced by MN fractions prepared from clinically isolated strains of C. albicans KYUP#3 and C. krusei KYUP#35. MN fractions were prepared under 2 culture conditions, C-limiting and YPD media, using clinically isolated strains of KYUP#3 and KYUP#35. MN fractions (1 mg) dissolved in PBS were administered to DBA/2 mice (n = 7) for 5 consecutive days, and survival was observed 150 days after the last day of administration of the MN fraction. CAWS (1 mg) was used as positive and negative control. Three weeks after the last administration of MN fractions, 2 mice in each group were sacrificed. Body and heart weights were measured, and HE-stained sections of the aorta were prepared.
suggest that the vasculitis activity of the MN fractions of clinical isolated Candida may be different and characteristic to species, strain, or medium. Furthermore, in the preliminary screening, several clinically isolated strains of Candida species were cultured in both YPD medium and C-limiting medium; however, non-albicans Candida species yielded smaller quantities of MN fractions, and none of the strains of Candida glabrata could be grown in C-limiting medium. We therefore collected clinically isolated Candida species and examined their vasculitis activities. MN fractions may be prepared from clinical isolates using different methods with different culture media and culture temperatures. The purpose of this study was to clarify the species as well as strain dependency of vasculitis induction in Candida. Using in vivo animal model for screening required sufficient quantities of polysaccharide fractions, thus, we used hot water extraction. In the present study, we selected YPD medium to prepare MN fractions because of its higher yield (CKY35 vs. CKC35) and stronger activity (CAY3 vs. CAC3).

Preparation of MN fractions from clinically isolated Candida species

We collected clinically isolated strains of Candida from the stocks in Kyorin University Hospital. Stock cultures were collected from specimens of venous blood, catheters, central venous catheters (CVC), blood sampling from CVC, bile, or sputum. The outcomes of patients infected with each strain varied and are not described in the present study. Due to different frequencies of clinical isolation, the number of specimens varied depending on the species; the percentages of C. albicans and C. parapsilosis in particular were high. To normalize the number of test samples, we set the maximum number of each species to 5. We tested the following 26 strains: C. albicans (5 strains), Candida dubliniensis (3 strains), C. glabrata (5 strains), Candida guilliermondii (2 strains), C. krusei (1 strain), C. parapsilosis (5 strains), and C. tropicalis (5 strains). These strains were cultured in YPD medium, and MN fractions were prepared using the methods described above. Yield and physicochemical properties are shown in Table 1.
Survival of MN fraction-administered mice

Each MN fraction (4 mg) was i.p. administered to DBA/2 mice for 5 consecutive days, and survival was monitored every day until day 200 (Fig. 3, Tables 2, 3, 4). Heart weights were measured when mice died or on the last day of the experiment (Table 4).

As described above (Figs 1 and 2), the death of mice during the experimental period was attributed to severe lethal vasculitis. All CAWS-administered mice died as expected (mean survival days: 78.6 ± 51.9). Death rates by Candida species were as follows: *C. albicans* (84%), *C. dubliniensis* (47%), *C. glabrata* (32%), *C. guilliermondii* (20%), *C. krusei* (100%), *C. parapsilosis* (44%), and *C. tropicalis* (20%) (Table 3).

### Table 1. Strains used in this study and physicochemical properties of MN fractions

| Isolate           | Abbreviations | Specimen* | Yield (mg) | Component (%) | Elemental analysis | Residue | C | H | N |
|-------------------|---------------|-----------|------------|---------------|-------------------|---------|---|---|---|
| *C. albicans*     | CAY13         | CVC       | 271.5      | 754.6         | 40.7 6.5 8.9       | 71.9    | 19.4 |
| *C. albicans*     | CAY14         | Catheter  | 262        | 624.5         | 40.3 6.5 9         | 88.7    | 18.7 |
| *C. albicans*     | CAY21         | Blood     | 216.8      | 538.1         | 38.5 6.3 8.5       | 80.3    | 19.0 |
| *C. albicans*     | CAY34         | Blood     | 250.4      | 691.5         | 39.3 6.5 8.7       | 55.2    | 31.6 |
| *C. albicans*     | CAY37         | Blood-c   | 380.8      | 1014          | 41.5 6.4 8.6       | 78.6    | 19.5 |
| *C. dubliniensis* | CDY17         | CVC       | 291.1      | 1012          | 41.9 6.3 11.1      | 50.4    | 18.0 |
| *C. dubliniensis* | CDY51         | Bile      | 314.1      | 758.9         | 39.6 6.4 9.2       | 46.2    | 29.3 |
| *C. dubliniensis* | CDY52         | Sputum    | 211.5      | 652.6         | 40.3 6.6 9.4       | 54.8    | 34.5 |
| *C. glabrata*     | CGY9          | Blood     | 252.1      | 623.4         | 42.3 6.5 9.6       | 42.4    | 31.0 |
| *C. glabrata*     | CGY26         | Blood     | 361        | 706           | 40.6 6.5 8.6       | 46.2    | 26.4 |
| *C. glabrata*     | CGY29         | Blood     | 311.8      | 647.5         | 38.4 6.4 8.2       | 52.1    | 20.2 |
| *C. glabrata*     | CGY41         | Blood-c   | 270.2      | 782.5         | 39.4 6.7 8.7       | 51.4    | 24.8 |
| *C. glabrata*     | CGY46         | Blood     | 333.5      | 880.8         | 38.4 6.5 8.5       | 49.0    | 22.3 |
| *C. guilliermondii| CGUY5         | Blood-c   | 165        | —             | 40.1 6.4 8.8       | 41.9    | 32.6 |
| *C. guilliermondii| CGUY32        | Blood     | 174.5      | 390           | 38.4 6.4 9.1       | 35.7    | 29.0 |
| *C. krusei*       | CKY35         | CVC       | 302.3      | 693           | 41.8 6.2 8.8       | 40.9    | 23.9 |
| *C. parapsilosis* | CPY2          | Blood     | 250.4      | —             | 42.3 6.6 8.7       | 42.8    | 22.0 |
| *C. parapsilosis* | CPY11         | Blood     | 193.3      | 427.8         | 41.1 6.4 9         | 25.4    | 24.7 |
| *C. parapsilosis* | CPY12         | CVC       | 226.8      | 588.8         | 42.2 6.4 10.1      | 56.2    | 37.5 |
| *C. parapsilosis* | CPY43         | Blood     | 182.8      | 744.5         | 39.7 6.7 9.1       | 58.0    | 36.8 |
| *C. parapsilosis* | CPY48         | Blood-c   | 304.5      | 522.1         | 37.5 6.7 7.8       | 41.2    | 22.5 |
| *C. tropicalis*   | CTY7          | CVC       | 224.6      | 593           | 40.5 6.2 11.1      | 42.2    | 30.7 |
| *C. tropicalis*   | CTY16         | Blood     | 217.9      | 656.6         | 40.3 6.7 6.2       | 76.4    | 21.2 |
| *C. tropicalis*   | CYT18         | CVC       | 185.7      | 561.2         | 47.8 7.3 8.2       | 38.2    | 28.5 |
| *C. tropicalis*   | CTY27         | Blood     | 174.5      | 471.4         | 38.6 6.2 9.1       | 32.0    | 22.8 |
| *C. tropicalis*   | CTY39         | CVC       | 208.9      | 448           | 40.2 6.1 11.1      | 27.3    | 28.4 |

* A Blood; Venous blood, CVC; Central venous catheter, Blood-c; Blood sampling from CVC

*B From 400 ml culture by YPD medium at 37°C

- Not done

### Table 2. Statistical difference of lethal vasculitis induced by MN fractions of Candida spp.

|   | *C. albicans* | *C. dubliniensis* | *C. glabrata* | *C. guilliermondii* | *C. tropicalis* | *C. krusei* | *C. parapsilosis* | *C. tropicalis* |
|---|--------------|------------------|-------------|-------------------|----------------|-------------|-------------------|----------------|
|   | <0.001       | <0.001           | <0.001     | <0.001            |               | <0.001     | <0.001            |               |
| *C. dubliniensis* | <0.05    | <0.05            |           |                   |               |            |                   |               |
| *C. glabrata*     | <0.001    |                   |            |                   |               |            |                   |               |
| *C. guilliermondii| <0.001   |                   |            |                   |               |            |                   |               |
| *C. tropicalis*   |           |                   |            |                   |               |            |                   |               |
| *C. krusei*       |           |                   |            |                   |               |            |                   |               |
| *C. parapsilosis* |           |                   |            |                   |               |            |                   |               |
| *C. tropicalis*   |           |                   |            |                   |               |            |                   |               |
Fig. 3. Survival of mice administered MN fractions of clinically isolated *Candida* species.

Each MN fraction shown in Table 1 (4 mg/mouse) was administered i.p. to DBA/2 mice (n = 5) for 5 consecutive days. Survival, body weight, and heart weight were monitored for 200 days. On the last day, surviving mice were sacrificed, heart weights were measured, and HE-stained sections of the aortic region were prepared. Each figure includes one *Candida* species shown on the top and CAWS. Each solid line shows survival days (%) of each strain. Each figure contains 5 solid lines except for *C. dubliniensis* (3 lines), *C. guilliermondii* (2 line), and *C. krusei* (1 line). Dotted line shows that for CAWS. Numbers shown in the right side of each line indicate strain of MN fraction.
Mean survival days were shorter with *C. albicans*, *C. dubliniensis*, *C. krusei*, and *C. parapsilosis* than with the other strains (Table 4). The species dependency is also obvious from the number of dead mice on day 100 (Table 3), wherein the total number of dead mice in each species were *C. albicans* (15/25), *C. dubliniensis* (5/15), *C. glabrata* (0/25), *C. guilliermondii* (0/10), *C. krusei* (5/5), *C. parapsilosis* (5/25), and *C. tropicalis* (1/25). These results strongly suggest that the severity of vasculitis is dependent on the species. In comparisons among species, the severity of vasculitis induced by *C. albicans* was significantly stronger than that by *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*, while that induced by *C. krusei* was significantly stronger than that by *C. dubliniensis*, *C. glabrata*, *C. guilliermondii*, *C. parapsilosis*, and *C. tropicalis* (Fig. 3, Table 2). Strain dependency was also analyzed based on the survival days of mice administered with each strain. Significant differences were observed among strains within *C. albicans*, within *C. parapsilosis* (CPY11 vs CPY43), and within *C. tropicalis* (CTY16 vs CTY18, CTY16 vs CTY27) (Table 3). Cardiomegaly is a characteristic feature of lethal vasculitis. As shown in Table 4, heart weights were higher in dead mice than in surviving mice, even for lower lethality groups (*C. glabrata*, *C. guilliermondii*, *C. parapsilosis*, and *C. tropicalis*).

Table 3. Strain differences in lethal vasculitis activity of MN fractions assessed by survival days

| Species             | Abbreviations | Number of dead mouse | Mean ± SD |
|---------------------|---------------|----------------------|-----------|
|                     |               | Day 100 | Day 200 |             |
| CAWS                |               | 4   | 5     | 78.6 ± 51.9 |
| *C. albicans*       | CAY13         | 2   | 4     | 118.2 ± 47.6 |
|                     | CAY14         | 5   | 5     | 63.6 ± 5.9  |
|                     | CAY21         | 1   | 3     | 155.8 ± 46.8 |
|                     | CAY34         | 4   | 4     | 99 ± 51.1   |
|                     | CAY37         | 3   | 5     | 90.2 ± 17.1 |
| *C. dubliniensis*   | CDY17         | 0   | 1     | 180.6 ± 38.8 |
|                     | CDY51         | 3   | 4     | 105 ± 54.3  |
|                     | CDY52         | 2   | 2     | 144 ± 68.6  |
| *C. glabrata*       | CGY9          | 0   | 2     | 187.2 ± 15.9 |
|                     | CGY26         | 0   | 1     | 196.8 ± 6.4 |
|                     | CGY29         | 0   | 3     | 173.4 ± 24.3|
|                     | CGY41         | 0   | 1     | 198 ± 4.0   |
|                     | CGY46         | 0   | 1     | 193.6 ± 12.8|
| *C. guilliermondii* | CGUY5         | 0   | 2     | 181.6 ± 33.4|
|                     | CGUY32        | 0   | 0     | 200 ± 0.0   |
| *C. krusei*         | CKY35         | 5   | 5     | 69.4 ± 21.6 |
| *C. parapsilosis*   | CPY11         | 3   | 4     | 110.6 ± 57.7|
|                     | CPY43         | 0   | 0     | 200 ± 0.0   |
|                     | CPY12         | 0   | 2     | 173.6 ± 32.5|
|                     | CPY48         | 1   | 2     | 156.6 ± 53.6|
|                     | CPY2          | 1   | 3     | 156.2 ± 47.2|
| *C. tropicalis*     | CTY16         | 1   | 3     | 150.6 ± 60.5|
|                     | CTY18         | 0   | 0     | 200 ± 0.0   |
|                     | CTY27         | 0   | 0     | 200 ± 0.0   |
|                     | CTY7          | 0   | 1     | 192.2 ± 15.6|
|                     | CTY39         | 0   | 1     | 198.6 ± 2.8 |

# Survival days for living mice were set to 200.

*; < 0.05, **; < 0.01, ***; < 0.005

4). Mean survival days were shorter with *C. albicans*, *C. dubliniensis*, *C. krusei*, and *C. parapsilosis* than with the other strains (Table 4). The species dependency is also obvious from the number of dead mice on day 100 (Table 3), wherein the total number of dead mice in each species were *C. albicans* (15/25), *C. dubliniensis* (5/15), *C. glabrata* (0/25), *C. guilliermondii* (0/10), *C. krusei* (5/5), *C. parapsilosis* (5/25), and *C. tropicalis* (1/25). These results strongly suggest that the severity of vasculitis is dependent on the species. In comparisons among species, the severity of vasculitis induced by *C. albicans* was significantly stronger than that by *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*, while that induced by *C. krusei* was significantly stronger than that by *C. dubliniensis*, *C. glabrata*, *C. guilliermondii*, *C. parapsilosis*, and *C. tropicalis* (Fig. 3, Table 2). Strain dependency was also analyzed based on the survival days of mice administered with each strain. Significant differences were observed among strains within *C. albicans*, within *C. parapsilosis* (CPY11 vs CPY43), and within *C. tropicalis* (CTY16 vs CTY18, CTY16 vs CTY27) (Table 3). Cardiomegaly is a characteristic feature of lethal vasculitis. As shown in Table 4, heart weights were higher in dead mice than in surviving mice, even for lower lethality groups (*C. glabrata*, *C. guilliermondii*, *C. parapsilosis*, and *C. tropicalis*).
Histology of the aortic region on day 200

In the present study, severe lethal vasculitis was monitored based on survival days and heart weights, and the results obtained revealed species and strain differences. For *C. krusei*, only one strain was collected from the hospital, and all mice tested died within 200 days (Figs 2 and 3, Tables 3 and 4); the data for tissue sections are shown in Fig. 1. Similarly, all mice treated with CAY3, CAY14, and CAY37 died within 200 days (Fig. 2, Table 3). However, it currently remains unclear whether vasculitis was induced in surviving mice. To examine vasculitis in more detail, we prepared tissue sections from some of the surviving mice and summarized the results in Fig. 4. The tissue sections of 16 out of 23 mice did not have inflammatory lesions around the aorta.

For *C. albicans*, 4 out of 5 mice died (Table 3) and the surviving mouse induced vasculitis; thus, strongly suggesting that all the tested mice induced vasculitis. In contrast, the tissue sections of CAY21 and CAY34 were free from vasculitis. Similarly, for CDY51 (*C. dubuliniensis*), 4 out of 5 mice died (Table 3) and the surviving mouse had induced vasculitis; thus, strongly suggesting that all the tested mice had induced vasculitis. However, for CDY17 and CDY52, it remains unclear whether vasculitis was induced in all the surviving mice.

For *C. glabrata*, none of the tissue sections (CGY9, 26, 29, 41, and 46) had induced vasculitis. This is consistent with the fact that only few mice administered with CGY died (Tables 3 and 4).

For *C. guilliermondii*, none of the tissue sections developed vasculitis. This is consistent with the fact that none of the mice administered with CGUY32, CPY43, or CTY27 (Table 3) died. In addition, these MN fractions did not induce acute lethal shock in ICR mice (dead/total: 0/3, 0/3, 0/3, respectively). For CGUY5, the tissue section developed vasculitis, and no mice died on day 100 (Table 3); suggesting that the activity is weak. In contrast, surviving mouse administered with CPY11 did not have induced vasculitis. However, 3 mice died on day 100, and death rate was statistically significant from CPY43 (Table 3); suggesting that the activity is strong. Data shown in Fig. 4 and Table 3 indicate that other MN fractions of *C. parapsilosis*, such as CPY12, CPY48, and CPY2, exhibit moderate activity. For CTY, 2 out of 5 tissue sections indicated inflammation, and only few mice died (Table 3); suggesting that the activity is weak. All the data shown in this section strongly suggest that vasculitis induced by MN fractions of *Candida* was significantly dependent on both species and strains.

Structure of mannan required for vasculitis

In the previous investigation, Shinohara et al. demonstrated that CAWS prepared at neutral pH (CAWS727) did not induce vasculitis in mice due to the presence of a significant amount of β-linked mannose residues11. To clarify the contribution of the β-linked mannose residues of MN fractions to the induction of vasculitis by clinically isolated strains, we performed an NMR analysis. As shown in Fig. 5, the spectra of MN fractions in each species were similar to each other and showed species-specific signal patterns. According to Shibata’s assignment, the anomeric signals of α-mannose and β-mannose residues were characteristic to species and appeared at approximately 5.6-4.9 and 5.0-4.7 ppm, respectively. Consistent with these results, all of the spectra contained signals assignable to α-mannan. Furthermore, many of the spectra also contained signals assignable to β-mannan. It is interesting to note that the spectrum of *C. krusei* did not contain β-mannan signals. The spectrum of *C. parapsilosis* showed signals at approximately 4.9 ppm that may be α-1,6-linked man signals, which are consistent with previous findings by Shibata et al.42, 44, 46. From

|                | Total no. of mice | Dead/Survived (%) | Mean survival days (dead mice) | Dead mouse (mg) | Survived mouse (mg) |
|----------------|------------------|------------------|---------------------|----------------|-------------------|
| CAWS           | 5                | 5/0 (100)        | 79 ± 55             | 296 ± 90       |                   |
| *C. albicans*  | 25               | 21/ 4 (84)       | 87 ± 29             | 381 ± 92       | 275 ± 40          |
| *C. dubuliniensis* | 15            | 7/8 (47)         | 78 ± 26             | 282 ± 117      | 263 ± 36          |
| *C. glabrata*  | 25               | 8/17 (32)        | 170 ± 18            | 347 ± 79       | 247 ± 25          |
| *C. guilliermondii* | 10           | 2/8 (20)         | 154 ± 39            | 338 ± 26       | 246 ± 22          |
| *C. krusei*    | 5                | 5/0 (100)        | 69 ± 22             | 331 ± 37       |                   |
| *C. parapsilosis* | 25           | 11/14 (44)       | 107 ± 38            | 299 ± 98       | 266 ± 25          |
| *C. tropicalis* | 25              | 5/20 (20)        | 141 ± 55            | 363 ± 90       | 300 ± 70          |

Heart weight was measured at the day of death.
Heart weight of survived mouse was measured on day 200.
the view point of strain-specific signals, the signals around 5.55 ppm were present in at least 9 spectra, and the signals around 5.1-5.2 ppm were present in 4 spectra. These results suggest the strain specificity of the structure of mannoprotein in *Candida* species. However, the precise assignment of each

| CGY9  | CPY2  | CTY7  |
|------|------|------|
| CGY26| CPY11 | CTY16 |
| CGY29| CPY12 | CTY18 |
| CGY41| CPY43 | CTY27 |
| CGY46| CPY48 | CTY39 |

| CAY13 | CDY17 | CGUY5 |
|------|------|------|
| CAY21| CDY51 | CGUY32 |
| CAY34| CDY52 |     |

Fig. 4. HE staining of the aortic area of surviving DBA2 mice. Rectangular symbols indicate vasculitis-positive sections.

Dectin-2 is a pattern recognition receptor for α-mannan, and

of the sugar residue could not be performed due to the use of crude MN fractions. The precise structural information required to induce vasculitis, therefore, currently remains unclear.
Fig. 5. $^1$H-NMR spectra of MN fractions of clinically isolated Candida species.

1D-$^1$H NMR spectra of MN fractions prepared from clinically isolated strains were measured. All spectra were recorded in D$_2$O at 343K using a Bruker Advance 500 spectrometer. Chemical shifts are reported in ppm relative to acetone-d$_6$ as an internal standard ($\delta H = 2.189$ ppm). Strain numbers are shown in the left side of each spectra. Numbers shown in the left side of each line indicate strain of MN fraction.
CAWS has been identified as a ligand for dectin-2. Oharaseki et al. recently demonstrated that dectin-2-deficient mice were resistant to CAWS-vasculitis. To examine the relationship between CAWS-2 binding and vasculitis, we examined the reactivity of MN fractions to recombinant dectin-2. As shown in Fig. 6, all MN fractions reacted with dectin-2. The relative reactivities of C. glabrata and C. guilliermondii to dectin-2 were significantly lower than those of the MN fractions of other species. Similarly, the vasculitis activity of these species was weak compared with other species (Fig. 3, Tables 2 and 3). In terms of strain specificity, CDY51 and CTY16 showed the strongest vasculitis activity (Table 3) as well as dectin-2 reactivity among the different species. These results also strongly suggest that reactivity to dectin-2 is crucial for the induction of vasculitis.

Discussion

We and others previously reported that CADS and CAWS induced vasculitis in mice. The resulting vasculitis resembled that in KD patients, and thus, this is a suitable animal model for examining the vasculitis of KD. CAWS-vasculitis, particularly in DBA/2 mice, is severe and ultimately results in death due to cardiac hypertrophy. Vasculitis is induced not only by the MN fractions of the standard strains of C. albicans, but also by those of C. metapsilosis and C. krusei. Knockout mice for Dectin-2, a PRR for fungal pathogens, are resistant to CAWS-vasculitis, suggesting that the activation of dectin-2 signaling by MN fractions is one of the key steps in the induction of vasculitis. Furthermore, MN fractions containing β-mannosyl linkages, those synthesized at approximately 27°C under neutral pH conditions, exhibited weak activity, suggesting negative signaling by β-mannose residues.

In the present study, we investigated whether clinically isolated Candida have similar pathophysiology. The MN fractions of clinically isolated Candida species were prepared, vasculitis-inducing activities were assessed using the most sensitive strain (DBA/2 mice), and the structural features previously demonstrated by the standard strains were examined. The results showed that 1) vasculitis was induced by several species of Candida and its severity differed depending on the species, i.e., more severe vasculitis was induced by C. albicans and C. krusei than by C. glabrata and C. guilliermondii. 2) The induction of vasculitis was dependent on the strains of Candida. 3) Vasculitis was dependent on binding affinity to dectin-2. These results were consistent with previous findings obtained using the standard strains.

We previously reported that CAWS727, prepared from the culture supernatant of C. albicans NBRC1385 cultured at 27°C for 2 days in pH-controlled C-limiting medium (pH 7), did not induce vasculitis, and this was attributed to the presence of β-mannosyl linkages. These findings were strongly supported by Shibata et al., who reported that β-mannosyl transferase was only active at approximately 27°C and neutral pH. In the present study, we compared the structures of MN fractions using NMR analysis and found that signals assignable to β-mannosyl linkages were detected in various species, including C. albicans, C. dubliniensis, C. glabrata, C. guilliermondii, and C. tropicalis. The higher ratio of β-mannosyl linkages may be a negative factor for clinically isolated Candida species; however, the relationship between vasculitis activity and the presence of β-mannosyl linkages currently remains unclear. Furthermore, some of the NMR spectra show strain-specific signals around 5.1 to 5.2 ppm, i.e., CAY21, CGU5, CPY11, and CPY48, suggesting the strain specificity of the structure of mannoprotein in Candida species. The purpose of this study was to clarify the species as well as strain dependency of vasculitis induction in Candida. We prepared MN fractions from clinically isolated Candida species and examined coronary vasculitis induced by these fractions. Using in vivo animal model for screening required sufficient quantities of MN fractions, thus, we used hot-water-extracted MN fractions. A more detailed structural examination of each strain using purified preparation of MN fractions is required for clarification of the relationship between structure and vasculitis activity.

Previous studies demonstrated the contribution of various factors to the induction of CAWS-vasculitis, including dectin-2 signaling, the induction of Th17 cells, activation of the IL-1 β pathway, changes in the microbiome, CCL2 production, CCR2-, CXCL9-, and CXCL10-dependent signaling, LPA-LPA1 signaling, the MBL-dependent complement pathway, and the contribution of the vasa vasorum. Dectin-2 dependency is consistent with mannan being a fungal PAMP and ligand for dectin-2. In the present study, we examined the direct binding of MN fractions to recombinant dectin-2 and found that relative binding activity significantly differed in a species-dependent manner. C. glabrata and C. guilliermondii exhibited significantly lower binding capacities than other species. In addition, CDY51 and CTY16 showed the strongest vasculitis activity (Table 3) as well as dectin-2 reactivity (Fig. 6) among different species. These results were strongly consistent with vasculitis being dependent on dectin-2 signaling.

In the present study, relative activities were dependent not only on species, but also on strains. As shown in Table 3, the relative activities of C. albicans significantly differed among CAY13, CAY14, CAY21, CAY34, and CAY37, with CAY14 exhibiting the strongest activity and CAY21 the weakest. A comparison of the NMR spectrum and dectin-2 binding revealed some differences. In preliminary experiments on
Fig. 6. Reactivities of MN fractions of clinically isolated Candida species to dectin-2.
Ninety-six-well half-area microplates (Greiner bio-one) were coated with CAWS or MN fractions of Candida species dissolved in bicarbonate buffer (pH 9.8). After blocking by 1.0% BSA/PBST (with Ca/Mg), serially diluted solutions of recombinant h-dectin-2 conjugated with the human Ig-Fc fragment (Sino Biological) were added (maximum concentration, 1.0 μg/mL). After the incubation, anti-human IgG-POX was added to detect recombinant dectin-2. After extensive washing, plate-bound dectin-2 was detected using TMB reagent. Each figure includes one Candida species shown on the top and CAWS. Each figure contains 5 solid lines except for C. dubliniensis (3 lines), C. guilliermondii (2 line), and C. krusei (1 line). Numbers shown in the right side of each line indicate strain of MN fraction. The dotted line (・・・) and solid line ( ■ ) show the results for CAWS and the blank, respectively.

Concentration of h-dectin-2
acute toxicity, death rates by CAY14 and CAY21 were 2/3 and 0/3 (dead/total), respectively. Kobayashi et al. and Okawa et al. demonstrated the strain dependency of the mannan structure of C. tropicalis in relation to culture conditions. Their findings strongly suggested that the mannan structure is controlled by various factors, including strain as well as culture conditions, which may influence vasculitis activity.

The incidence of candidiasis caused by non-albicans Candida species is increasing. These species are resistant to commonly used antifungal drugs, such as fluconazole. Azole resistance is also becoming more prevalent due to the use of related chemicals as pesticides. In the present study, coronary vasculitis was induced by the MN fractions of clinically related chemicals as pesticides. In the present study, coronary vasculitis activity.

Since vasculitis was induced even by non-albicans Candida, its induction is one of the pathological factors for Candida species.

Conflicts of interest

The authors declare no conflicts of interest.

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