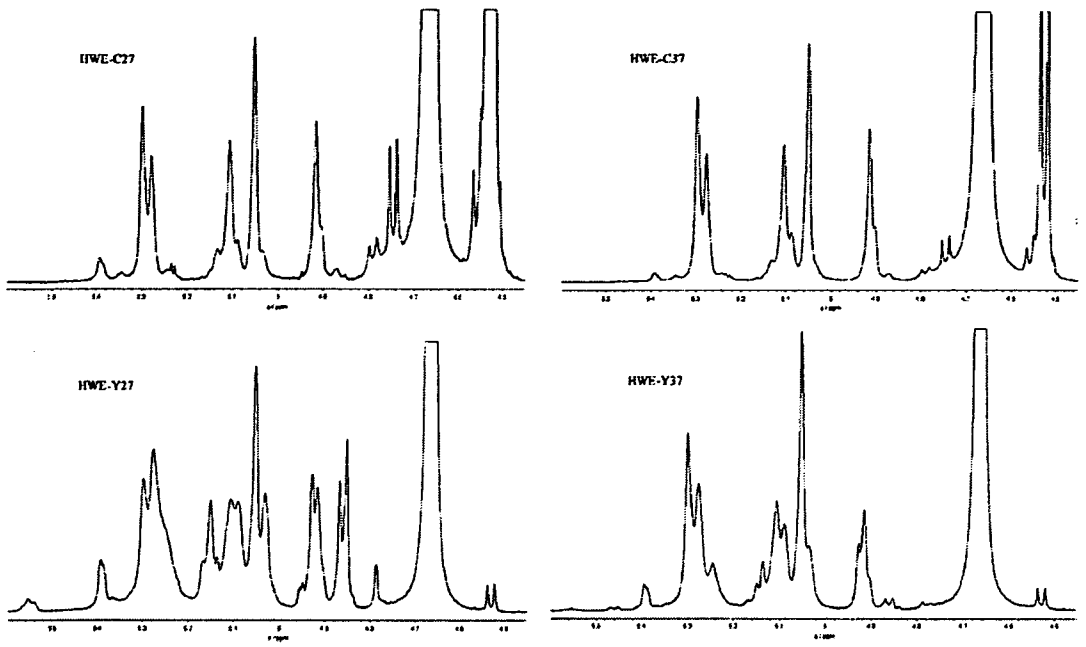

Fig. 3. 1D-^{1}H NMR spectra of cell wall extracts. 1D-^{1}H NMR spectra of hot water extracts of *C. albicans* NBRC 1385 derived from various culture conditions. All spectra were recorded in D_2O at 310 K using a Bruker Avance 500 spectrometer equipped with a TXI *xyz*-three gradient probe for ^{1}H detection. Chemical shifts are reported in ppm relative to acetone-d_6 as an internal standard ($\delta_H = 2.189$ ppm).

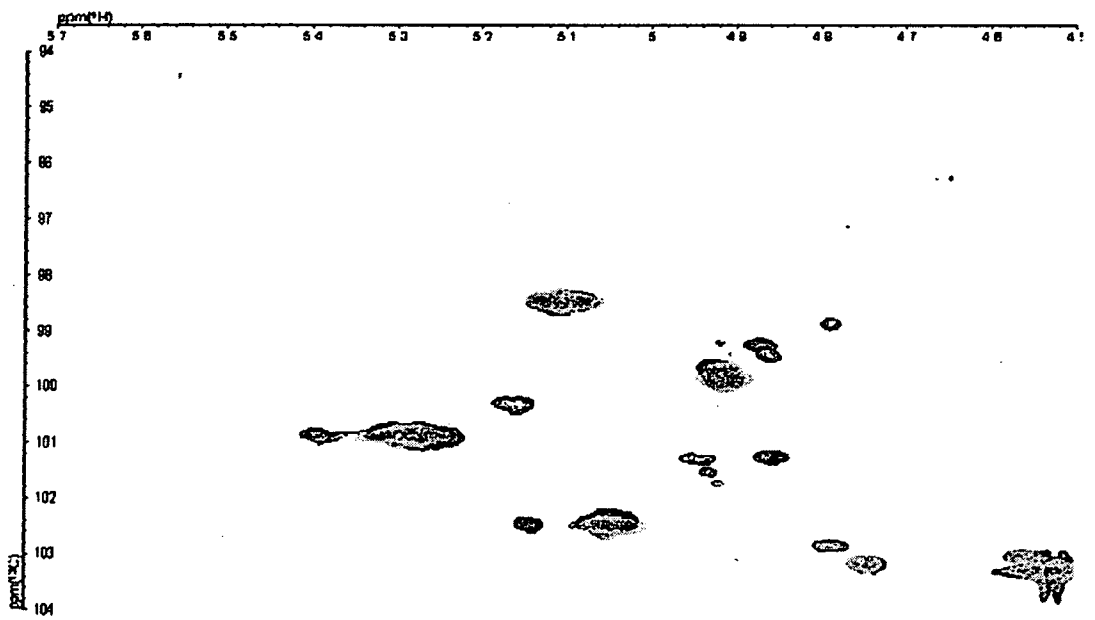

Fig. 4. Overlaid ^{1}H, ^{13}C-HSQC spectra of hot water cell wall extracts. The overlaid ^{1}H, ^{13}C-HSQC spectra from (black) HWE-C27, (green) HWE-C37, (red) HWE-Y27, and (blue) HWE-Y37 derived from *C. albicans* NBRC 1385. All spectra were recorded in D_2O at 310 K using a Bruker Avance 500 spectrometer equipped with a TXI *xyz*-three gradient probe for ^{1}H detection. Chemical shifts are reported in ppm relative to acetone-d_6 as an internal standard ($\delta_H = 2.189$ ppm, $\delta_C = 31.45$ ppm).

3. Discussion

In the present study, we clearly demonstrated that cell wall carbohydrates extracted from the pathogenic fungus *C. albicans* dramatically induced coronary arteritis similar to KS as well as acute anaphylactoid shock in mice. Moreover, these biological effects depended on environmental conditions around *Candida* cells including growth temperature and culture media. Alteration of the environmental conditions led to structural rearrangement of cell

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Table 5
Anomeric conformation analyses of hot water cell wall extracts of *Candida albicans* cultured in various culture conditions

	^{1}H (ppm)	^{13}C (ppm)	$^1J_{H1,C1}$ (Hz)	Conformation	Contain				Residue
					C27	C37	Y27	Y37	
1	5.56	94.5	172	α-Mannose			●		Manβ1→2Manα1→phosphate
2	5.54	94.5	174	α-Mannose			●		Manβ1→(2Manβ1)$_n$→2Manα1→phosphate
3	5.39	100.9	173	α-Mannose	●	●	●	●	α1→2Manα1→3Manα1→2
4	5.39	100.9	176	α-Mannose			●	●	α1→2Manα1→3Manα1→2
5	5.30	100.8	172	α-Mannose	●	●	●	●	Manα1→2Manα1→2
6	5.28	100.9	172	α-Mannose	●	●	●	●	α1→2Manα1→2Manα1→2
7	5.25	100.9	172	α-Mannose			●	●	α1→3Manα1→2Manα1→2Manα1→2 ↑6 Manα1
8	5.17	100.3	172	α-Mannose			●		Manβ1→2Manα1→2
9	5.15	100.3	172	α-Mannose			●		Manβ1→(2Manβ1)$_n$→2Manα1→2
10	5.15	102.5	171	α-Mannose				●	Manα1→3
11	5.14	102.5	172	α-Mannose			●	●	Manα1→3
12	5.13	98.4	173	α-Mannose	●	●			Manα1→3
13	5.11	98.5	172	α-Mannose	●	●	●	●	α1→6Manα1→6Manα1→6Manα1→6 ↑2 Manα1(→2Manα1)$_n$
14	5.09	98.5	170	α-Mannose	●	●	●	●	(→6Manα1→)$_n$ ↑2 Manα1(→2Manα1)$_n$
15	5.05	102.5	172	α-Mannose	●	●	●	●	Manα1→2
16	5.04	102.4	171	α-Mannose	●	●	●	●	α1→3Manα1→2
17	4.95	101.3	162	β-Mannose			●		Manβ1→2Manβ1→2Manβ1→2Manβ1→2Manα1→2
18	4.93	101.3	162	β-Mannose			●		Manβ1→(2Manβ1)$_n$→2Manα1→2
19	4.93	101.5	162	β-Mannose			●		Manβ1→(2Manβ1)$_n$→2Manα1→2
20	4.92	101.7	160	β-Mannose			●		Manβ1→(2Manβ1)$_n$→2Manα1→2
21	4.93	99.6	160	β-Mannose			●		Manβ1→(2Manβ1)$_n$→2Manα1→2
22	4.93	99.7	171	α-Mannose				●	Manα1→6
23	4.92	99.9	172	α-Mannose	●	●	●	●	Manα1→6
24	4.90	99.8	170	α-Mannose	●	●	●	●	Manα1→(6Manα1)$_n$→6Manα1→6
25	4.92	99.2	160	β-Mannose			●		Manβ1→2Manβ1→2Manβ1→2Manα1→2
26	4.87	99.2	159	β-Mannose			●		Manβ1→2Manβ1→2Manα1→2
27	4.86	99.4	160	β-Mannose			●		Manβ1→2Manβ1→2Manα1→phosphate
28	4.86	101.2	162	β-Mannose			●	●	Manβ1→2Manβ1→2Manα1→2
29	4.84	101.2	160	β-Mannose			●		Manβ1→2Manβ1→2Manα1→2(3)
30	4.79	98.9	159	β-Mannose			●		Manβ1→2Manα1→2
31	4.78	102.8	163	β-Glucose	●	●			
32	4.74	103.2	162	β-Glucose	●	●			
33	4.56	103.1	163	β-Glucose	●	●			
34	4.53	103.3	162	β-Glucose	●	●	●	●	

wall carbohydrates, especially mannan. On the basis of these findings, *Candida* cell wall mannan might contribute to coronary arteritis and acute shock, and an alteration of mannan structure could be responsible for *Candida* pathogenicity.

The cell wall extracts used in this study were mainly composed of carbohydrates (mannose and glucose) and protein, with no endotoxin contamination (Table 1). Moreover, *Candida* cell wall components extracted using the hot water method dramatically induced coronary arteritis (Figs. 1 and 2) and acute anaphylactoid shock in mice (Table 2). Since these effects were retained after changing the extraction technique to the alkaline reflux method (data not shown), the carbohydrates within the cell wall extracts are likely the components responsible for the biological activities evaluated in this study. We ruled out the importance of the O-linked mannosyl residue, since alkaline reflux would cause β-elimination of the O-linked oligosaccharides [29]. It is therefore likely that *Candida* cell wall carbohydrates, especially N-linked carbohydrates, could be at least partially responsible for pathogenic induction of coronary arteritis and acute anaphylactoid shock.

The present study also found that only the cell wall extract derived from YPD medium at 27 °C (Y27) did not exhibit any biological effects (Figs. 1, 2, and Table 2). This result strongly suggests that environmental conditions (not only culture temperature but also medium) are crucial for pathogenicity of *C. albicans*. To understand the difference in biological effects, we examined the mannan structures of the extracts because *Candida* cell wall mannan is well known to contribute to its antigenicity and pathogenicity

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Table 6
Ability of mild acid hydrolyzed HWE to induce rapid anaphylactoid shock in ICR mice

Dose (mg/kg)	Anaphylactoid shock		
	Incidence	Score[a]	Mortality
C27			
0	0/4	0	0/4
4	0/4	0	0/4
8	4/4	3–4	2/4
16	4/4	4	3/4
C37			
0	0/4	0	0/4
4	2/4	1	0/4
8	4/4	2–4	1/4
16	4/4	4	4/4
Y27			
0	0/4	0	0/4
4	0/4	0	0/4
8	2/4	2–3	0/4
16	3/4	3	0/4
Y37			
0	0/4	0	0/4
4	2/4	2–4	1/4
8	3/4	2–4	2/4
16	3/4	3–4	1/4

Indicated dose (mg/kg) was i.v. administered to mice ($n = 4$). Mortality was monitored within 1 h. */*, number of mice, dead/total.

[a]The scoring of the shock was as follows: 0, no symptoms of shock; 1, staggering; 2, crawling and prostration; 3, prostration and weak convulsions; 4, prostration and strong convulsions.

and can also be altered by environmental conditions such as growth temperature [25], pH [26], and osmotic pressure. As revealed by the reactivity of *Candida* serum factors (Table 3), the reactivity of cell wall extracts prepared using various culture conditions was significantly different. The specific epitopes of sera nos. 5 and 6 (Table 4) only reacted with cell wall extracts from Y27. These results were also confirmed by NMR data (Figs. 3 and 4). Further precise structural elucidation by the method of Shibata et al. [27] using 2D-TOCSY spectra showed that only cell wall extracts from Y27 contained additional signals for β-mannosyl residues within both acid-stable and acid-labile regions (Table 5). In addition, to examine the involvement of β-1,2-linked mannooligosaccharides in anaphylactoid shock in mice, we evaluated the effect of mild acid hydrolysis, which cleaves the phosphodiester linkage of the acid-labile region. However, we were not able to observe any modification of biological effect (Table 6). Therefore, these data suggest that the specific epitope of sera nos. 5 and 6 within the acid-stable region that only reacted against cell wall extracts from Y27 may prevent the induction of acute anaphylactoid shock and coronary arteritis. This also implied that both biological effects might be triggered by essentially similar mechanism(s). Namely, the effects might progress followed by the recognition of α-mannan via mannan recognition molecules; however, the analyses of the precise mechanism(s) are still needed, e.g. the affinity of cell wall mannan against mannan lectins. Since α-mannan residues that only contained bioactive mannan could not be identified in this study, β-1,2-linkage might cover α-mannnosyl residues as most mannan-binding proteins can sense the nonreducing terminal α-mannosyl residue. Therefore, the lectin complement pathway might not be activated. However, the possibility of a direct negative action of β-1,2-linked mannooligosaccharides still remains.

We also examined the possibility of the involvement of molecular weight differences (data not shown) in biological effects. However, we did not find any relationship between biological effects and molecular weight distribution.

Numerous studies of the antigenicity and pathogenicity of fungal cell wall mannans, especially those from *C. albicans* and *Saccharomyces cerevisiae*, have been reported. Kind et al. [30] reported that the lethal toxicity and increased vascular permeability of some yeast mannans, including *C. albicans*, appear to depend on the α-1,2-, α-1,6-linkage in their main chain. Garner et al. [31] reported that TNF-α is produced in vivo in response to mannan derived from *C. albicans*. These effects can be regulated by mannan ligands such as anti-mannan antibodies and corticosteroids. Moreover, numerous studies have shown that β-1,2-linked mannan, which is only expressed by pathogenic yeasts such as *C. albicans*, is vital for cell adhesion to host cells [22–24] and cytokine production from various cells [20,21]. This specific glycan does not bind to typical mannan receptors such as the macrophage mannose receptor (MMR) or mannose-binding lectin (MBL). However, some studies have recently reported that galectin-3 is the receptor for β-1,2-linked mannan [32,33], and may contribute to some biological effects of mannan [34,35].

Our laboratory previously reported that CAWS, an extracellular polysaccharide fraction obtained from the culture supernatant of *C. albicans* [11], could induce coronary arteritis and acute anaphylactoid shock [12,13,15]. These biological effects depended on the pH of the culture process [16]. CAWS synthesized in neutral pH conditions that resulted in the expression of β-1,2-mannosyl residues significantly reduced acute anaphylactoid shock, coronary arteritis, and complement activation. This pattern of results was matched by the results from the present study. In the present study, we clearly demonstrated that only the β-mannosyl residue attached to nonreducing terminal α-mannosyl branched chains within an acid-stable region was very different between biologically active and inactive mannan. These findings are a significant advance compared with previous reports. Notably, in this study we showed that culture temperature and media as well as pH influenced the biological effects. However, little information about the influence of culture conditions on the biological effects of cell wall mannan is currently available. Most previous studies of mannan biology did not consider the culture conditions. Our study

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indicates that culture conditions should be considered when examining the biological effects of mannan.

The present study suggests that the pathogenic fungus *C. albicans* may contribute to coronary arteritis such as that observed during KS and sepsis caused by fungi. We found that these biological effects were susceptible to the environmental conditions around the cells; altered conditions led to structural rearrangement of the mannan components. Since the expression of β-1,2-linked mannan was dramatically different between biologically active and inactive mannan, β-1,2-linked mannan might inhibit *Candida* cell wall extract-induced coronary arteritis and acute anaphylactoid shock in mice. However, the *Candida* cell wall extracts used in this study were contaminated by β-glucan and protein. Thus, preparation of highly purified mannan and an evaluation of its biological effects is still needed. Nevertheless, these findings still suggest the possibility of a novel strategy for drug therapy: regulation of the biosynthesis of *Candida* cell wall mannan could be a candidate for therapy of coronary arteritis and acute anaphylactoid shock.

4. Materials and methods

4.1. Animals and materials

Male ICR and DBA/2 mice (6 weeks old) were purchased from Japan SLC. The mice were housed in a specific pathogen free (SPF) environment. All animal experiments followed the guideline of laboratory animal experiments in the Tokyo University of Pharmacy and Life Sciences (TUPLS), and each experimental protocol was approved by the committee of laboratory animal experiments at TUPLS. The completely synthetic medium, C-limiting medium [36] contained (per liter): sucrose 10 g, $(NH_4)_2SO_4$ 2 g, KH_2PO_4 2 g, $CaCl_2 \cdot 2H_2O$ 0.05 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $ZnSO_4 \cdot 7H_2O$ 1 mg, $CuSO_4 \cdot 5H_2O$ 1 mg, $FeSO_4 \cdot 7H_2O$ 0.01 g, and biotin 25 μg (final pH, 5.2). The natural medium, YPD medium, contained (per liter): yeast extract 10 g, Bacto peptone 20 g, D-glucose 20 g (final pH, 5.2). .*Candida* Check was from Mitsubishi Kagaku Iatron.

4.2. Yeasts and culture conditions

C. albicans NBRC 1385 was obtained from the National Institute of Technology and Evaluation Biological Resource Center (NBRC). *C. albicans* was grown as follows: 4 L of a completely synthetic medium, C-limiting medium, or a natural medium, YPD medium, was added to a fermenter and yeasts were cultured for 2 d at 27 °C or 37 °C with air supplied at a rate of 5 L/min. Following the culture, an equal volume of ethanol was added to kill yeasts. Cells were then extensively washed with distilled water and acetone dried. Acetone-dried cells were further delipidated with $CHCl_3$–MeOH (2:1, v/v; 600 mL) for 2 h under reflux. The residual cells were then washed with EtOH (600 mL) for 2 h under reflux to remove low-molecular-weight organic compounds.

4.3. Preparation of hot water extract

The washed cells (20 g) were suspended in 1 L of distilled water and then autoclaved for 4 h at 128 °C. This suspension was allowed to cool to room temperature (RT). After centrifugation, the supernatant was carefully collected. The residual sediment was re-extracted two times by the methods described above. The combined supernatants were concentrated at 40 °C in vacuo to a volume of about 250 mL. After centrifugation for the removal of traces of insoluble material, the water extract was dialyzed against distilled water. After centrifugation, the water solution was lyophilized.

4.4. Preparation of alkaline extract

The washed cells (20 g) were suspended in 200 mL of 10% aqueous KOH and then extracted for 2 h under reflux under an N_2 atmosphere. This suspension was allowed to cool to RT. After centrifugation, the supernatant was carefully collected. The residual sediment was re-extracted two times by the methods described above. The combined supernatants were neutralized by HCl and then concentrated at 40 °C in vacuo to a volume of about 250 mL. After centrifugation for the removal of traces of insoluble material, the water extract was dialyzed against distilled water. After centrifugation, the water solution was lyophilized.

4.5. Sugar analysis

Polysaccharides were completely hydrolyzed in 2.0 M CF_3CO_2H (115 °C, 1.5 h). The sugars were converted to alditol acetates by reduction followed by treatment with acetic anhydride in an equal volume of pyridine (100 °C, 1 h), and then analyzed by GC using a GC-2014AF instrument (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a 30 m × 0.25 mm (0.25 mM) DB-225 capillary column (J and W Scientific, CA, USA).

4.6. Other chemical analyses

Total carbohydrate concentration was determined by the phenol–sulfuric acid method using a mixture of D-mannose and D-glucose as a standard. Total protein was determined by the BCA Protein Assay Regent kit (PIERCE biotechnology), using bovine serum albumin as a standard. Endotoxin content was determined by the Toxicolor LS-50M Set (Seikagaku Corporation).

4.7. Administration schedule for induction of coronary arteritis [12]

We used the DBA/2 mouse strain in this experiment because this strain showed the most serious coronary

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arteritis after treatment with CAWS, which is secreted into the culture supernatant from *C. albicans*. Cell wall extracts (4 mg/mouse) were administered intraperitoneally for 5 consecutive days to each mouse in week 1. The hearts of the animals were fixed with 10% neutral formalin and embedded in paraffin blocks. Tissue sections were stained with HE. Preparation of paraffin blocks and HE staining was done by Japan SLC.

4.8. Scoring of rapid anaphylactoid shock

The incidence and the severity of rapid anaphylactoid shock were assessed within 1 h of i.v. injection (0.1 mL/10 g body weight) of cell wall extracts into ICR mice. These values and the subsequent mortality (in the first hour after injection) were recorded. The scoring of shock was as follows: 0, no symptoms of shock; 1, staggering; 2, crawling and prostration; 3, prostration and weak convulsions; 4, prostration and strong convulsions.

4.9. Enzyme-linked immunosorbent assay (ELISA) of the reactivity to Candida serum factors

The reactivity of cell wall extracts to serum factors from *Candida* Check, which consists of rabbit polyclonal antibodies against *Candida* cell wall mannan [37,38] was detected by ELISA. The epitope of the serum factors were previously reported as described in Table 4 [39]. A solution of cell wall extracts in 50 mM carbonate buffer (pH 9.6) was coated onto Nunc immunoplates, which were then incubated at 4 °C overnight. The plates were washed extensively with 0.05% Tween 20 containing phosphate-buffered saline (PBST); unbound sites were blocked by the addition of 1% BSA containing PBST (BPBST) to wells for 40 min at 37 °C and then the wells were washed 6 times with PBST. *Candida* serum factors serially diluted with BPBST were added and incubated for 60 min at 37 °C. After 6 washes with PBST, the wells were treated with peroxidase-conjugated goat anti-rabbit IgG and the 3,3′,5,5′-tetramethylbenzidine microwell peroxidase substrate system (TMB; KPL Inc.). After 45 min, the reaction was stopped with 1 N H_3PO_4, and then the optical density of each well was read at 450 nm on an automatic microplate reader.

4.10. NMR spectroscopy

Exchangeable protons were removed by dissolving cell wall extracts in D_2O, and samples were then lyophilized. This exchange process was repeated three times. All NMR spectra were recorded in D_2O at 310 K using a Bruker Avance 500 spectrometer equipped with a TXI *xyz*-three gradient probe for ^1H detection. Chemical shifts are reported in ppm relative to acetone-d_6 as an internal standard ($\delta_H = 2.189$ ppm, $\delta_C = 31.45$ ppm). Data processing was performed using XWinNMR software. The 1D-^1H experiment was performed using a Bruker standard pulse sequence with 4310 Hz in 64 K complex data points. The relaxation delay used was 5T1 in order to calculate accurate signal integrations. Prior to Fourier transformation, 4 times zero filling was used, and noise was reduced using the Trafication function. 2D sensitivity improvement ^1H, ^{13}C-HSQC without decoupling during acquisition was conducted to measure $^1J_{H1,C1}$ with 512 increments of 2048 data points with 32 scans per t_1 increment using the Bruker standard pulse sequence. The spectral width was 3501 Hz for t_2 and 12,500 Hz for t_1. 2D-TOCSY was conducted with a mixing time for TOCSY spinlock of 30–180 ms using the pulse sequence of Griesinger et al. [40] to suppress ROE signals. The spectral width was 2200 Hz in each dimension and 512 increments of 4096 data points with 16 scans per $t1$ increment were recorded. All 2D experiments were zero-filled to 2 and 2 k in both dimensions prior to Fourier transformation. A cosine-bell window function was applied in both dimensions.

4.11. Mild acid hydrolysis

Treatment of the samples with 10 mM HCl was conducted as described previously [28]. Briefly, samples were dissolved in 10 mM HCl, and the resulting solutions were heated in a boiling water bath for 1 h. The solutions were neutralized with 100 mM NaOH, and the hydrolysates were then ethanol precipitated (4:1).

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【成因・病態】

急性期動脈炎の病理組織像からみた川崎病の成因

Presumed causative agent of Kawasaki disease from histopathological view point

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◎川崎病の病理組織学的特徴は，冠状動脈をはじめ中型の臓器外筋型動脈に生じる単球/マクロファージの集積を主体とした増殖性肉芽腫性炎症と総括できる．本特徴は，同様の大きさの血管が侵襲されるがフィブリノイド壊死をみ，臓器内血管にも病変が生じる結節性多発動脈炎や，小児に好発し大動脈などに感染リンパ球浸潤をみるEBウイルス関連血管炎とは大きく異なる．疫学，臨床データは川崎病の発症に感染因子が関与していることを示しており，近年も細菌由来のスーパー抗原やある種のウイルスが候補としてあげられているが，いずれも検出頻度の川崎病優位性には問題が残る．病理組織像の違いは成因，病態の差を表している可能性が高く，単球/マクロファージの異常活性化をもたらす何らかの感染因子が川崎病発症に関与していると推測される．

Key word：川崎病，病理組織像，冠状動脈炎，単球/マクロファージ，増殖性肉芽腫性炎症

成因論概略

疫学，臨床データは何らかの感染因子が発症に関与していることを強く示唆しているが，川崎病の病因はいまだ不明である．川崎病発見から今日に至る40年間に提唱された成因仮説を列挙すれば，ダニ，水銀，合成洗剤などの非感染説にはじまり，細菌(溶連菌，緑連菌，サンギス菌，ブドウ球菌，エルシニア，プロピオン酸菌など)，ウイルス(ロタ，EB，RS，HHV-6，レトロウイルスなど)，リケッチアによる感染説など，きわめて多岐に及ぶ[1]．しかし，微生物の分離同定や血清学的検索，そして他の動物への接種などさまざまな手法を用いても確認されていないのは周知のとおりである．

近年注目された成因仮説として，まずスーパー抗原[2]がある．スーパー抗原はT細胞表面のTCR Vβ領域と単球/マクロファージなど抗原提示細胞のα鎖とに結合し，抗原非特異的に，MHC class II分子の個体差に関係なくT細胞を活性化，増殖させる．急性期川崎病にみられる免疫反応の著しい亢進，著明な高サイトカイン血症は，ブドウ球菌や溶連菌などに由来するスーパー抗原(TSST-1，SPA，SPC，SPEA，SPECなど)による刺激の結果として矛盾しないと考えられた．しかし，さまざまなスーパー抗原が成因候補として示されたにもかかわらず，スーパー抗原産生菌検出についての川崎病優位性はどれも疑問符がついたままである．

ついで，2005年新種のコロナウイルス(HCoV)が川崎病罹患児から高率に分離されたとの報告がなされた[3]．しかし，このウイルスはHCoV NL-63としてすでに同定されているものと同一であることが確認され，さらに，つぎつぎに行われた追試験はすべて否定的な結果に終わっている[4]．

Rowleyらは，川崎病剖検例の血管病変部にIgA形質細胞とCD8リンパ球が多数の単球/マクロ

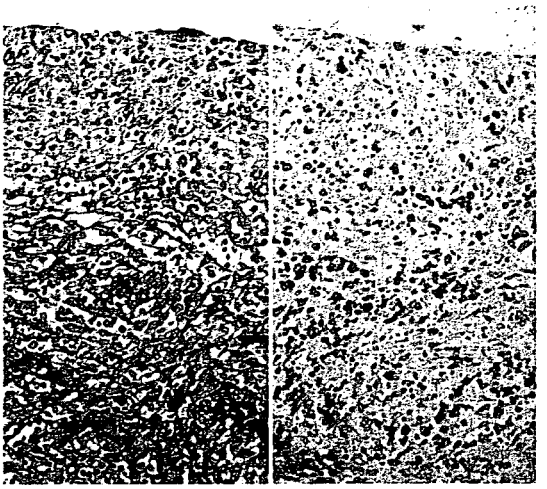

図 1　川崎病冠状動脈炎(24 病日死亡例)
内弾性板，中膜平滑筋層など既存の動脈構築は著しく破壊され，CD68 陽性単球/マクロファージを主体とする細胞が血管壁の全層にわたって浸潤する．

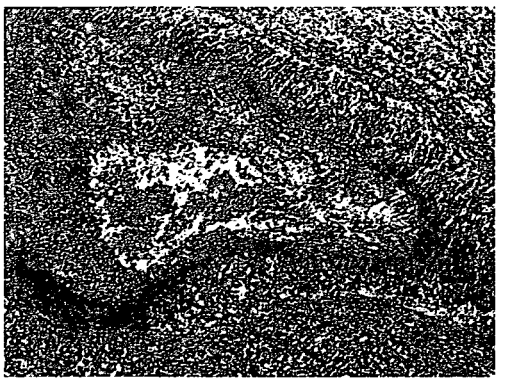

図 2　川崎病腎動脈炎(28 病日死亡例)
冠状動脈と同様の増殖性肉芽腫性炎症が観察される．

ファージとともに浸潤していることを示した．ついで，IgA 形質細胞は血管病変部のみでなく気管支や膵，腎などにも浸潤しており，さらに，IgA は oligoclonal な反応であることを報告した．これらのことから Rowley らは，何らかの細胞内病原因子が経気道的に侵入し，抗原特異的な反応が引き起こされていると仮定した[5]．そして川崎病血管病変組織から得た免疫グロブリンα鎖，κ鎖の可変領域遺伝子をクローニングし，ベクターに導入して合成抗体を作製した．この抗体を用いて川崎病剖検標本に対して免疫組織学検索を試みたところ，血管病変部に浸潤する単球/マクロファージや気管支の線毛上皮細胞などに抗体と反応する物質を確認した[6]．とくに，気管支線毛上皮細胞の細胞質に見出された球状の封入体様構造物は HE 染色でも微細な褐色顆粒として認識可能で，RNA ウイルスの存在が推測されたが，電顕的検索ではウイルス粒子や nucleocapsids の同定に成功していない．現在，成因解析に向けさらなる検討が続けられている．著者らの施設に保管されている日本人の川崎病剖検症例も，その一部は Rowley のもとに送られ研究対象に加えられている．川崎病の病因を呼吸器系ウイルスとするユニークな仮説であるが，細胞内感染因子に対する組織反応，川崎病血管炎には本当に CD8 優位のリンパ球浸潤が存在するのかという点にはじまり，経気道的に侵入した微生物が冠状動脈はじめ系統的血管炎を惹起させるメカニズムに至るまで，今後，検証・解明されなければならない点は多い．

川崎病血管炎の病理組織像の特徴

川崎病血管炎は発症 6 日頃にはじまり，10 病日ごろ汎血管炎に至る．その後，速やかに動脈全周の炎症に進展し，動脈の拡張がはじまる．動脈を構成する弾性板や中膜などの既存構築は著しく破壊され，肉芽腫性増殖性炎症と表現される単球/マクロファージの著明な浸潤がみられる(図 1)[7]．好中球やリンパ球，形質細胞も混在するが，比較的少数であり，フィブリノイド壊死をみることもまれである[7,8]．冠状動脈がもっとも高頻度に侵襲されるが，他にも腎や膵，肺など全身の動脈に炎症が生じる(図 2)．実質臓器の場合，臓器の外側に分布する動脈に炎症が生じるが，臓器内動脈に炎症は基本的に生じない．さらに，全身の血管病変はほぼ同様の推移を示し，新旧病変の混在はない．

他の血管炎疾患との比較

Jennette らの分類[9]にも示されているように，川崎病と同様の中型の筋型動脈が侵襲される疾患には結節性多発動脈炎(PN)がある．わが国における推定患者数はおよそ 1,400 名であるが，その大部分は 60 歳以上の高齢者であり，小児に限定すればきわめてまれな疾患といえる．過去には HB ウイルスの関与が指摘されたが，川崎病と同様，本疾

患の原因は明らかになっていない．しかし，川崎病とは明らかに異なる臨床像，病理組織像を呈し，成因や血管炎発生機序も大きく異なると推測される．PNにおける組織学的特徴の第1は，Arkin分類のⅠ期，Ⅱ期に相当するフィブリノイド壊死を伴った壊死性血管炎であり，著明な好中球浸潤を伴う．第2の特徴はPNでは同一臓器内に瘢痕期病変と急性炎症期病変とが混在する点で(図3)，川崎病のように一峰性の炎症経過をとらない．病変分布に関するとPNの好発侵襲臓器は腎，膵，肝，消化管，皮膚などであり，冠状動脈も侵襲されるが，その頻度は川崎病ほど高くない．さらに，PNでは川崎病とは異なり臓器外動脈に加え臓器内動脈も同時に侵襲される．

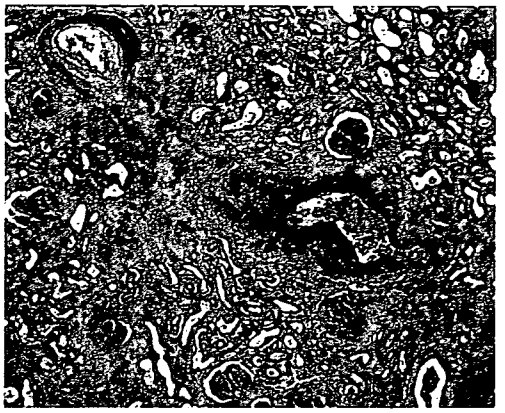

図3　結節性多発動脈炎(9歳，腎)
フィブリノイド壊死を伴う急性期炎症(右下)と瘢痕期動脈病変(左上)とが混在して認められる．AM染色．

全身性エリテマトーデス(SLE)や関節リウマチ(RA)などの膠原病に基づく血管炎も，小児にはまれな疾患であるが，血管病変を伴う場合には冠状動脈が侵襲されることが多い．血管壁には免疫複合体の沈着をみることが多く，フィブリノイド壊死を伴った血管炎が基本像であり，より小型の動脈が侵襲される傾向にある．

一方，小児に好発し因果関係が明確な動脈炎としてEBウイルス関連血管炎がある．慢性活動性EBウイルス感染症の診断基準を満たした患児に心血管病変が合併するまれな病態であり，発熱，肝脾腫，貧血，リンパ節腫大などに悪性リンパ腫や血球貪食症候群を合併し，その多くは発症後数年の経過で死亡する．大動脈に加え総頸動脈，総腸骨動脈，冠状動脈など大動脈の主要分枝に拡張や動脈瘤がもたらされる．病理組織学的には，高度の線維性内膜肥厚，中膜平滑筋細胞の巣状壊死や弾性線維の虫くい状の破壊がみられる(図4)．外膜栄養血管の周囲や中膜にはTリンパ球を主とする炎症細胞浸潤が認められ，このTリンパ球にEBウイルスの感染が確認される．感染Tリンパ球がサイトカイン産生や炎症細胞を活性化させ血管障害を引き起こしているものと推測される[10]．川崎病と比較すると好発年齢がやや高く臨床像は大きく異なる．冠状動脈は侵襲されるが，病変の

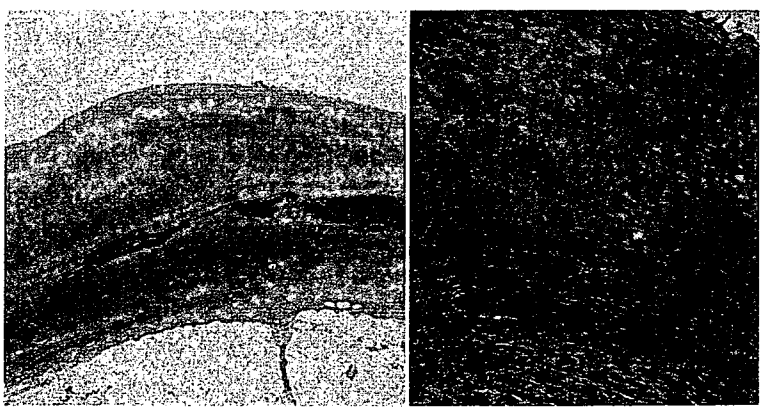

図4　EBウイルス関連血管炎(10歳，大動脈)
著しい内膜肥厚とともに中膜弾性線維は虫くい状に消失している．中膜平滑筋は巣状に消失する．本例ではリンパ球浸潤はほとんど認められない．左：EvG染色，右：HE染色．

主体は大動脈であること，浸潤細胞がリンパ球優位であることなどが川崎病との鑑別点となる．

おわりに

川崎病成因仮説の概要を述べ，病理組織像から小児血管炎疾患を比較した．血管炎疾患における病理組織像の差は同時に，成因や発症機序が異なることを示しているといえる．病理組織学的見地からも，単球/マクロファージの異常な活性化をもたらす因子が川崎病発症に関与していると推測される．しかし，単球/マクロファージを活性化させるさまざまな因子のなかから，病理組織像のみで川崎病の成因をみつけだす作業は至難といわざるをえない．

たとえば，著者らが検索を継続しているカンジダに由来する物質を用いたマウス系統的血管炎誘発モデルでは冠状動脈炎が高頻度に惹起され，病変部には多数の単球/マクロファージが出現する[11]．本モデルで得られる血管炎の病理組織像や病変分布は，*Lactbacillus casei* や異種血清によって誘発される血管炎モデルと比較し明らかに川崎病に類似する．本モデルで血管炎を惹起させているのはカンジダ菌体から培養液中に溶出した可溶性mannoprotein-β-glucan complex であり，この成分が単球/マクロファージやリンパ球を活性化させ炎症性サイトカインを放出し，血管炎に至ると考えられる[12]．このように本物質は，すくなくともマウスにおいて単球/マクロファージを活性化させ血管炎を引き起こす．しかし，川崎病と同じ症状がマウスにも出現しているのかという点については現時点では確認できていない．成因を特定するためには異なる原因が結果として近似した組織像を示す血管炎を引き起こす可能性をも念頭におき，多面的に解析していく必要がある．

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Kawasaki Disease Arteritis and Polyarteritis Nodosa

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Abstract: Arteritis is a common feature of Kawasaki disease and coronary artery involvement is the major cause for mortality. The mucocutaneous lymph node syndrome is the defining feature of Kawasaki disease. Therefore, although the pathologic features of acute Kawasaki disease are very distinctive, this disease should be diagnosed only in an appropriate clinical setting. The acute phase of Kawasaki disease arteritis occurs almost exclusively in young children: however, approximately 5% of patients have chronic coronary artery sequelae. This should be kept in mind when evaluating coronary artery disease in older children and adults, especially if there are features of a chronic aneurysm. The presence or absence of the mucocutaneous lymph node syndrome is the most definitive feature for differentiating Kawasaki disease arteritis from polyarteritis nodosa occurring in a child. Kawasaki disease arteritis typically has less fibrinoid necrosis in the acute phase than polyarteritis nodosa. Based on histology alone, the arteritis of polyarteritis nodosa is indistinguishable from arteritis that may occur as a component of a small vessel vasculitis, such as microscopic polyangiitis or Wegener's granulomatosis.

Key Words: Kawasaki disease, polyarteritis nodosa, systemic arteritis, coronary arteritis

(*Pathology Case Reviews* 2007;12: 193–199)

CASE REPORT

A 9-month-old male infant was taken to a hospital on the 3rd day of illness, with chief complaints of a fever and rash. He was immediately hospitalized because of findings of systemic polymorphous exanthema, bilateral bulbar conjunctival injection, edema of the hands and soles, and cervical lymphadenopathy. Erythema of the lips manifested on the 4th day of illness, and a diagnosis of Kawasaki disease was made. Aspirin was administered. However, the fever persisted, and on the 5th day of illness all of the principal symptoms of Kawasaki disease were met. Thus, high-dose intravenous immunoglobulin (IVIG) was administered. The fever decreased on the 7th day of illness, and ultrasonography performed on that day revealed no abnormalities of the coronary arteries. However, the fever recurred on the 9th day of illness, and ultrasonography performed on the 14th day revealed dilation of the coronary arteries. Accordingly, a second dose of IVIG was given on the 15th day. Ultrasonography performed on the 16th day showed an aneurysm at the origin of the right coronary artery having a diameter reaching 4.3 mm. Early in the morning of the 18th day, the patient suddenly began vomiting and developed bradycardia, facial pallor, and shock. Electrocardiography revealed ST elevation in leads II, III, and aVF. Immediately, cardiopulmonary resuscitation was performed and urokinase drip infusion was started, but the patient died.[1]

Pathology

Postmortem examination revealed 2 aneurysms at the origin of the right coronary artery having a diameter of up to 5 mm. The aneurysms were filled with thrombi (Fig. 1). The left coronary artery was slightly dilated, but there was no aneurysm. Histologic inspection revealed all layers of the coronary artery walls to have a high degree of inflammatory cell infiltration consisting mainly of monocytes/macrophages and lymphocytes. The right coronary artery was dilated like a balloon, and the arterial wall had extreme thinning, with inflammatory cell infiltration accompanied by disruption of the internal and external elastic lamina and loss of the smooth muscle layer of the tunica media (Fig. 2). The aneurysms were filled with thrombus, and there was evidence for acute myocardial ischemia distributed throughout the posteroinferior wall of the left ventricle. Various degrees of inflammation were noted in arteries throughout the body, including the subclavian artery, common iliac artery, renal artery, interlobar arteries of the kidney, splenic artery, mesenteric artery, etc.[2]

DISCUSSION

Kawasaki Disease

Kawasaki disease was first reported in 1967 by Dr. Tomisaku Kawasaki as mucocutaneous lymph node syndrome, a disease of unknown etiology most commonly occurring in infancy and early childhood.[3,4] Kawasaki disease is a systemic vasculitis that invades primarily medium-sized muscular arteries.[5] The highest incidence of involvement is of the coronary arteries. Death from Kawasaki disease is most frequently attributable to ischemic heart disease in children

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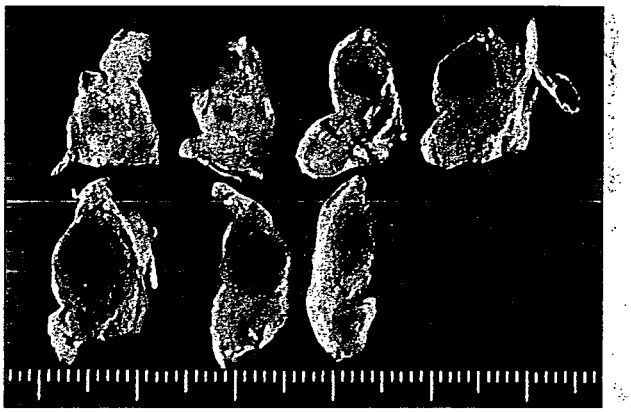

FIGURE 1. Gross appearance of aneurysms in the right coronary artery. The aneurysms are filled with thrombus.

caused by thrombosed coronary artery aneurysms, secondary to coronary arteritis.[6]

The age distribution of onset of Kawasaki disease shows a peak at 9 to 11 months, and 70% of all cases occur before the age of 3 years.[7] The male-to-female ratio among patients with Kawasaki disease is approximately 1.4:1. Although Kawasaki disease is markedly more prevalent in Japan and in children of Japanese ancestry, children of nearly all racial backgrounds are affected. Disease onset in siblings is 1% of patients, and the recurrence rate is 4%. Today, the mortality rate has fallen to only 0.04%.[7] Acute-stage cardiac abnormalities occurring within 1 month after disease onset show an incidence of about 15%, and the incidence of cardiac sequelae lasting at least 1 month is about 5%. The principal cardiac abnormality is coronary artery dilation during both the acute and remote stages.[7]

Diagnosis of Kawasaki disease is based on clinical signs and symptoms, which are classified as principal clinical findings and other clinical and laboratory findings.[8–10] The principal symptoms are: (1) fever persisting for 5 days or more (inclusive of those cases in whom the fever subsides before the 5th day in response to therapy), (2) bilateral conjunctival congestion, (3) changes in the lips and oral cavity, eg, reddening of the lips, strawberry tongue, diffuse injection of the oral and pharyngeal mucosa, (4) polymorphous exanthema, (5) changes in the peripheral extremities, eg, reddening of the palms and soles, and indurative edema in the initial stage, and membranous desquamation of the fingertips in the convalescent stage, and (6) acute nonpurulent cervical lymphadenopathy in the convalescent stage. At least 5 of these 6 criteria must be satisfied for a diagnosis of Kawasaki disease; however, patients having 4 of the principal symptoms can be diagnosed as having Kawasaki disease when a coronary aneurysm or dilation is detected by 2-dimensional echocardiography or coronary angiography.

In 1984, Furusho et al postulated treatment of Kawasaki disease by IVIG.[11] Since then, the efficacy of this treatment has become widely recognized,[12,13] and today immunoglobulin is administered to 85% of children with acute-stage Kawasaki disease.[7] IVIG has drastically reduced not only the incidence of coronary artery disease but also mortality due to Kawasaki disease.[7,14] In about 20% of patients IVIG does not show a good therapeutic effect, with 15% of patients developing transient dilation or other acute-stage coronary artery complications.[7,15–17] Various other treatments have been tried for patients who do not respond to IVIG, including steroids,[18,19] neutrophil elastase inhibitors,[20,21] plasma-exchange therapy,[22] and anticytokine antibody therapy.[23]

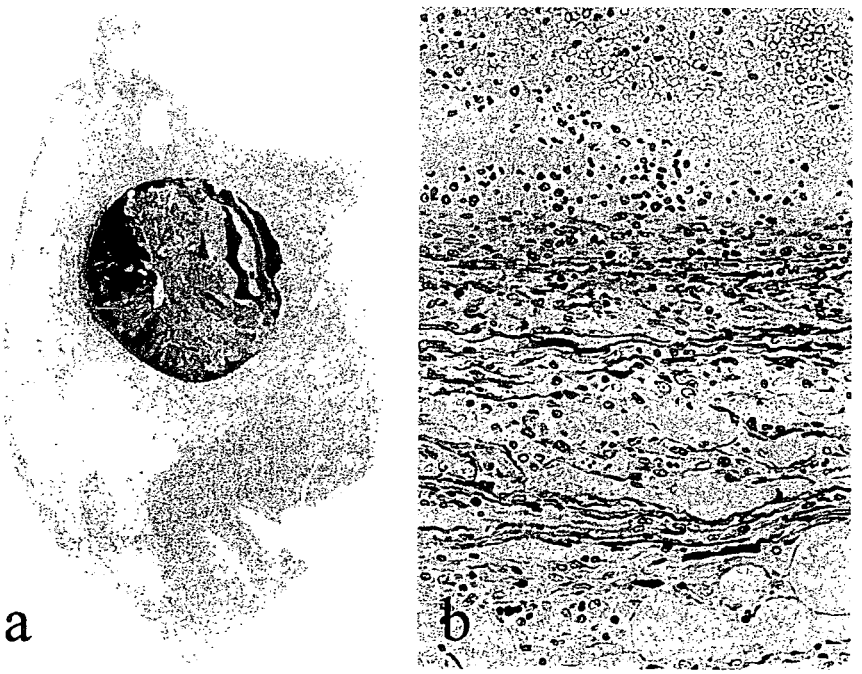

FIGURE 2. a, Low-power view of right coronary artery aneurysm. b, High-power view of aneurysm demonstrating inflammatory cell infiltration accompanied by destruction of the arterial wall (Elastica-van Gieson stain).

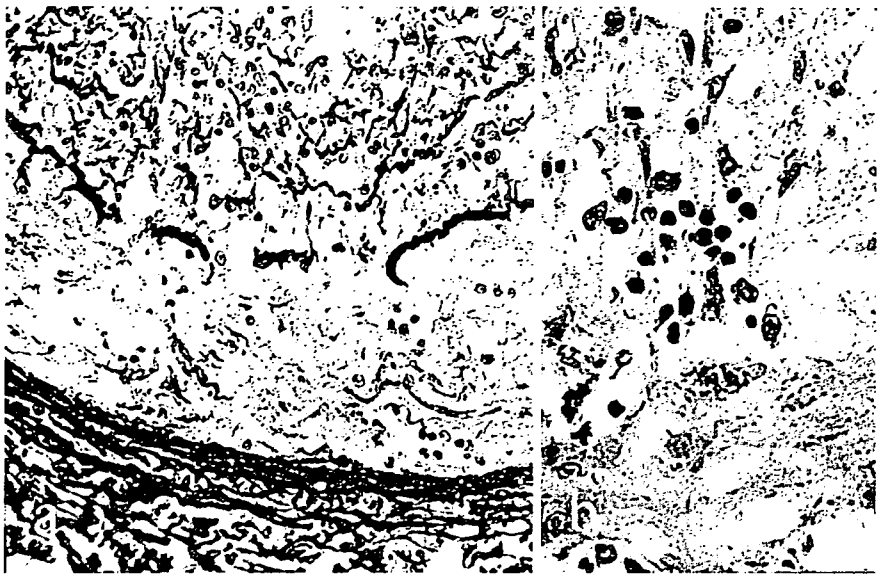

FIGURE 3. Coronary artery of Kawasaki disease patient who died 10 days after onset. a, Medium-power view of panvasculitis with disruption of internal elastic lamina (Elastica-van Gieson stain). b, High-power view, mononuclear leukocytes invade the site of disruption of internal elastic lamina.

The pathogenesis of Kawasaki disease involves activation of cells of innate and adaptive immunity, such as monocytes/macrophages and lymphocytes. These cells synthesize and secrete inflammatory cytokines and chemokines such as tumor necrosis factor-α, interferon-γ, interleukin-6, and monocyte chemotactic protein-1 (MCP-1), which activate immune cells and also activate endothelial cells.[24–28] As a result, adhesion molecules such as ICAM-1 and selectin are expressed in the endothelial cells,[29] which mediate leukocyte adherence to endothelial cells and migration into vascular walls where they cause damage to endothelial cells and smooth muscle cells. In addition, during the acute stage of Kawasaki disease there are increases in vasoactive substances, including growth factors such as vascular endothelial growth factor and platelet derived growth factor, as well as endothelin and nitric oxide,[30–33] which induce proliferation of intimal smooth muscle cells and increasing increased vascular permeability.

The pathologic changes that occur in Kawasaki disease arteritis can be broadly divided into the following stages: (1) initiation of arteritis 6 to 8 days after onset, (2) aneurysm formation 8 to 12 days after onset, (3) persistent arterial inflammation for 2 to 6 weeks after onset, and (4) scarring stage more than 6 weeks after onset.[34–38]

In Kawasaki disease autopsy cases that we have examined, 6 days is the shortest duration from the onset of the disease until death. In the patient reported above, the coronary arteries had slight infiltration of the tunica intima and the tunica adventitia by lymphocytes and monocytes/macrophages. However, the disruption of the internal elastic lamina was limited to an extremely small region, with no inflammatory cell infiltration of the tunica media. This suggests that Kawasaki disease coronary arteritis begins with endarteritis and periarteritis around the 6th day after onset of the disease.[39]

In patients who died on the 10th day of illness, inflammatory cells had caused panarteritis with infiltration of leukocytes in the coronary arteries that extended from the tunica intima and the tunica adventitia, across the internal and external elastic lamina. The internal elastic lamina showed multiple ruptures and there was damage to the smooth muscle cells of the tunica media, but there was no artery dilation (Fig. 3). The infiltrating inflammatory cells were identified immunohistologically, and most were found to be CD68+ monocytes/macrophages. Staining with antineutrophil elastase antibody also detected a considerable number of neutrophils in the tunica media (Fig. 4a,b). The infiltration of monocytes/macrophages and lymphocytes peaked around the 17th day of disease, whereas the highest level of neutrophil infiltration occurred at an earlier stage, around the 10th day.[39] The portion of smooth muscle cells in the media that had been infiltrated by the inflammatory cells showed edematous dissociation, and there was disappearance of the actin filaments and the basement membrane collagen of smooth muscle cells (Fig. 4c,d). On the basis of these findings, it can be postulated that enzymes such as metalloproteinases, elastases, and others, that are produced and secreted by neutrophils and monocytes/macrophages damage the endothelial cells and smooth muscle cells, and then damage the intercellular matrix and other vascular wall structural components.[39–41]

As the panarteritis advances, the internal elastic lamina, media, and other components that are crucial for maintaining the arterial structure undergo severe damage. As a result, from about the 12th day of disease, aneurysms may form and often contain thrombi. Aneurysms commonly form at the origins of the coronary arteries and the branching points of arteries (Fig. 5), which may be caused by hemodynamic factors at these locations.

The coronary artery in the acute stage of Kawasaki disease shows striking infiltration by monocytes/macrophages, lymphocytes, and neutrophils; however, fibrinoid necrosis is rarely observed in Kawasaki disease arteritis, which differs from the frequent finding of prominent fibrinoid necrosis in active polyarteritis nodosa. Severe inflammation

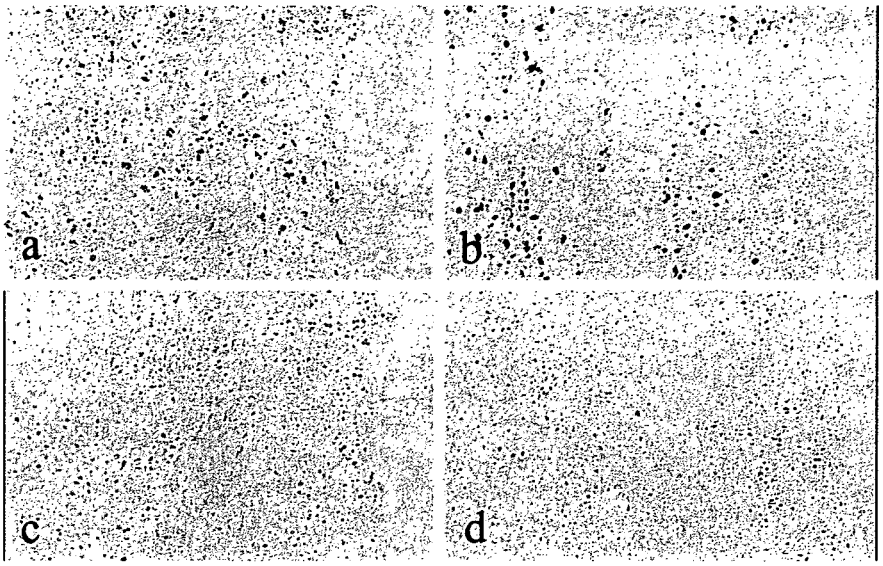

FIGURE 4. Immunohistochemical staining of coronary artery of Kawasaki disease patient who died 10 days after onset. a, CD68 for monocyte/macrophages. b, neutrophil elastase. c, α-smooth muscle actin. d, type IV collagen. The inflammatory cells were mainly composed of CD68+ monocytes/macrophages in the vascular wall. In addition, numerous neutrophils were also identified in the coronary arterial lesions of this patient. The damage of smooth muscle cells and intercellular matrix were observed in the same site as the inflammatory cell infiltration.

FIGURE 5. Diagram depicting the distribution of coronary artery aneurysms in Kawasaki disease patients.[33]

persists through about the 25th day of disease, after which it gradually subsides; by the 40th day the inflammatory cell infiltration typically has almost totally disappeared.[34–36] Some patients die during the acute stage of Kawasaki disease due to myocarditis and/or valvular disease, including a small number of patients with coronary arteritis but no aneurysms.

The inflammatory cell infiltration in Kawasaki disease arteritis almost completely disappears by about the 40th day of disease, but scarring remains for an extended period. This may include persistence of large fibrotic aneurysms with recanalization of thrombi. Remodeled chronically injured vessels can result in thrombotic occlusion even in the remote stage of Kawasaki disease[42–44] (Fig. 6). Thus, for patients in whom a large aneurysm forms during the acute stage, countermeasures will be required to prevent ischemic heart disease.[45]

Pathologic studies have shown that there is residual scarring from the arteritis even in the case of coronary arteries that are clinically judged to have returned to the normal state after there had been aneurysm formation or transient dilation.[46,47] Although such arteries may appear normal, there may be residual postinflammatory changes that are potential risk factors for atherosclerosis. This is an issue requiring careful study in the future.

Patients with Kawasaki disease develop cardiac lesions other than coronary arteritis. Autopsies of patients who died during the acute stage of Kawasaki disease show that the myocardium also has a high incidence of inflammatory cell infiltration. From about the 25th day after onset, inflammatory infiltration consisting mainly of monocytes/macrophages is seen in the interstitium of the myocardium (acute interstitial myocarditis). This inflammation may affect the conducting system.[48] Postmortem examination of patients who die acutely often reveals cardiac valvulitis.[49]

Polyarteritis Nodosa

Polyarteritis nodosa is another arteritis that involves medium-sized arteries and must be differentiated from Kawasaki disease.[5] Whereas most Kawasaki disease patients are children, polyarteritis nodosa occurs mostly in adults and differs a great deal in clinical course, prognosis, and treatment.

In Kawasaki disease arteritis there is involvement not only of the coronary arteries but also the arteries in many other tissues, including kidney, lung, ovary, testis, and mesentery.[50,51] Polyarteritis nodosa also involves arteries in many tissues, such as kidney, liver, gastrointestinal tract,

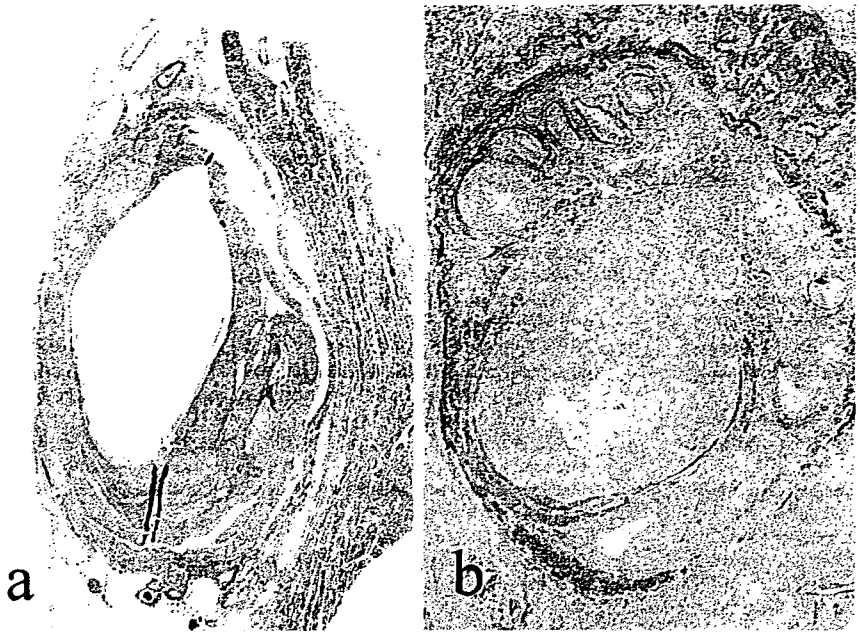

FIGURE 6. a, Coronary artery aneurysm of Kawasaki disease patient who died 17 years after onset. Dilation of lumen and lamellar calcification in the aneurysm wall. b, Recanalized vessels after thrombotic occlusion of aneurysm in a patient 4 years after the onset of Kawasaki disease.

nerves, skeletal muscle, skin, and heart.[52] However, whereas in Kawasaki disease the arteritis predominantly affects arteries before they enter the parenchyma of an organ, polyarteritis nodosa affects arteries both before and after they enter the parenchyma of an organ.[52] The histologic stage of lesions tends to be different between Kawasaki disease arteritis and polyarteritis nodosa. In Kawasaki disease, the lesions in all the arteries are relatively synchronous as they evolve from acute to chronic injury.[34–36] On the other hand, in polyarteritis nodosa, acute inflammatory lesions (Arkin's classification stages I and II) and chronic lesions (Arkin's classification stages III and IV) often are seen in the same organs at the same time.[52] In addition, in Kawasaki disease arteritis the acute inflammatory lesion is characterized by predominantly monocytes/macrophages and edema, usually without conspicuous fibrinoid necrosis,[34,53] whereas acute polyarteritis nodosa is characterized by predominantly neutrophil infiltration usually with prominent fibrinoid necrosis[52,54] (Fig. 7).

The relationship between Kawasaki disease arteritis and so-called infantile polyarteritis nodosa has been problematic. Many reported cases of infantile polyarteritis nodosa cannot be pathologically differentiated from Kawasaki disease arteritis,[55,56] although some cases appear to be example of polyarteritis nodosa occurring in young children.[56] The presence or absence of the mucocutaneous lymph node syndrome and the character of the histologic lesions are helpful in distinguishing between Kawasaki disease arteritis and polyarteritis nodosa occurring in a child.

Kawasaki disease arteritis and polyarteritis nodosa must be distinguished pathologically from necrotizing arteritis that occurs as a component of a small vessel vasculitis, such as microscopic polyangiitis or Wegener's granulomatosis.[5,57] In a pathology specimen, such as a muscle or nerve biopsy, the histopathology of arteritis caused by polyarteritis nodosa is indistinguishable from arteritis caused by microscopic polyangiitis, Wegener's granulomatosis or another small vessel vasculitis that can affect arteries. Thus, a definitive diagnosis of polyarteritis nodosa can not be made based on identification of arteritis in a biopsy specimen in the absence of additional information about the patient. In a patient with necrotizing arteritis, clinical or pathologic evidence for glomerulonephritis or pulmonary alveolar capillaritis rules out polyarteritis nodosa and indicates some from of small vessel vasculitis.[5,57]

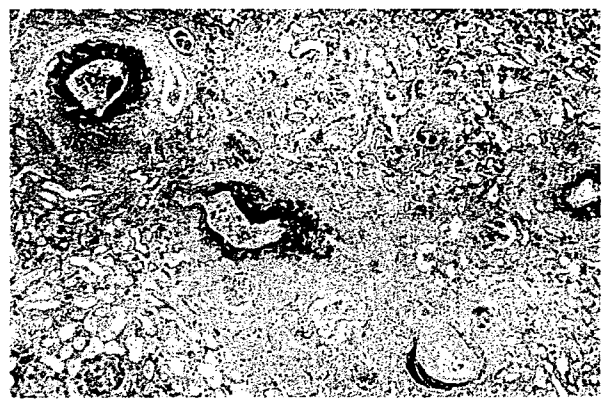

FIGURE 7. Arterial lesions in a 9-year-old girl with polyarteritis nodosa. Not the presence of both acute lesions with prominent fibrinoid necrosis and chronic sclerotic lesions. (Masson trichrome stain).

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Focused Microarray Analysis of Peripheral Mononuclear Blood Cells from Churg–Strauss Syndrome Patients

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Abstract

DNA diagnostics are useful but are hampered by difficult ethical issues. Moreover, it cannot provide enough information on the environmental factors that are important for pathogenesis of certain diseases. However, this is not a problem for RNA diagnostics, which evaluate the expression of the gene in question. We here report a novel RNA diagnostics tool that can be employed with peripheral blood mononuclear cells (PBMCs). To establish this tool, we identified 290 genes that are highly expressed in normal PBMCs but not in TIG-1, a normal human fibroblast cell. These genes were entitled *PREP* after predominantly expressed in PBMC and included 50 uncharacterized genes. We then conducted *PREP* gene-focused microarray analysis on PBMCs from seven cases of Churg–Strauss syndrome (CSS), which is a small-vessel necrotizing vasculitis. We found that *PREP135* (coactosin-like protein), *PREP77* (prosaposin), *PREP191* (cathepsin D), *PREP234* (*c*-fgr), and *PREP136* (lysozyme) were very highly up-regulated in all seven CSS patients. Another 28 genes were also up-regulated, albeit more moderately, and three were down-regulated in all CSS patients. The nature of these up- and down-regulated genes suggest that the immune systems of the patients are activated in response to invading microorganisms. These observations indicate that focused microarray analysis of PBMCs may be a practical, useful, and low-cost bedside diagnostics tool.

Keywords: focused microarray; RNA diagnosis; PMBC; allergic granulomatosis angiitis; Churg–Strauss syndrome

1. Introduction

The advent of array technology and the subsequent development of high-density oligonucleotide arrays[1] have been enormously helpful in improving our understanding of the genome-wide transcriptional profiles of many biological systems in both basic and applied research.[2] Array technology has also been extremely useful for discovering and developing diagnostic gene markers for disease subcategories, disease prognosis, and treatment outcome; this has paved the way for effective pharmaceutical drug discovery, the development of novel strategies for molecular (DNA, RNA) diagnostics, and the design of personalized drug regimens.[2]

Oligonucleotide microarrays, which were initially designed to analyze genome-wide gene expression levels, have turned out to be particularly useful in DNA diagnostics as they can be used for many different

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applications, including discovering polymorphisms and genotyping patients by identifying inheritable genetic markers.[3] With regard to the latter applications, such DNA diagnostics have greatly improved our understanding of and ability to detect the causative mutations of various diseases. However, this technology is hampered by major ethical and privacy issues since the genome sequence of a person carries unchangeable private information whose discovery can affect the life of not only that person, but also to some extent their family members. The need to protect patient privacy upon DNA diagnostics testing and the practical problems this constitutes also imposes extra costs on the testing procedure. Moreover, many kinds of diseases are caused not only by the DNA polymorphism, but also by environmental status. These environmental factors can be reflected in the RNA status, but DNA diagnostics usually cannot provide enough information on the environments. Thus, the activity of the disease can be detected by RNA or protein level but not by DNA level.

These problems are not faced by RNA diagnostics, in which the genome-wide mRNA levels are monitored by oligonucleotide (or cDNA) microarrays. Ethical issues of the nature described above do not apply here because the information obtained from RNA diagnostics does not necessarily relate to the DNA sequence. Instead, this technology monitors the dynamic changes in gene activities, namely, the increased or decreased transcriptional (mRNA) levels in the samples provided by the individual. The transcriptional levels would vary in the same individual depending on the health of the individual. In this sense, DNA diagnostics is a static test of the genome, whereas RNA diagnostics is a dynamic test of the genome.

However, it is not possible to monitor all 44 000 kinds of mRNA species that are transcribed from the human genome for many samples at the same time. A novel way to circumvent this problem is to develop a 'focused array' in which a limited number of mRNAs are tested in a low-density array. In the present study, we developed a focused oligonucleotide (or cDNA) array for use with patient peripheral blood mononuclear cells (PBMCs). For this, we selected the genes that are predominantly expressed in normal PBMCs, as determined by stepwise subtractive hybridization[4] and by genome-wide cDNA microarray analysis. From the 290 'PBMC-focused' genes we identified, we can prepare the PBMC-focused cDNA array. We examined the expression levels of these *PREP* genes to analyze the PBMC RNAs obtained from patients suffering the autoimmune disease Churg–Strauss syndrome (CSS), which is an alternative name of allergic granulomatosis angiitis, because the autoimmune response of CSS patients is expected to disturb the expression levels of immune-related genes in PBMC. Indeed, we identified several genes whose expressions are markedly up- or down-regulated in all CSS patients tested. These observations suggest that this low-cost RNA diagnostics test is useful, practical, and can be used at the bedside.

2. Patients, Materials and methods

2.1. *Human subjects: patients and healthy controls*

Blood was obtained from eight healthy volunteers (four males and four females; aged 25–49 years) for the cDNA library preparation. Blood was also obtained from seven cases of CSS patients whose profiles are shown in Supplementary Table S1 and 18 healthy controls (six males and 11 females; aged 25–86 years) for focused microarray analysis. CSS patients were diagnosed according to the diagnostic criteria of the American College of Rheumatology.[5] This study was reviewed and approved by the Internal Review Board of the Research Institute for Microbial Diseases, Osaka University. In accordance with the requirements of the Board, a written informed consent was obtained from each participant before venous blood samples were obtained. Serum samples were consecutively obtained regardless of the patient's symptom, active, or inactive phase.

2.2. *Preparation of RNA*

The RNA of the PBMCs obtained from healthy volunteers was prepared as described previously.[6] Briefly, heparinized venous blood (10 mL) was mixed with an equal volume of 2% dextran/saline solution and incubated at room temperature for 30 min to precipitate the red blood cells. Total RNA was extracted from the PBMC pellet by adding guanidine–thiocyanate solution and the samples were used for cDNA library preparation and subtractive hybridization.[7] Total RNA was also prepared by acid guanidinium–phenol–chloroform extraction for the DNA microarray, northern blot, and RT–PCR analyses. In some experiments, total RNA was synthesized using Ribo Max kit (Promega, Madison, WI) from the PBMC cDNA library. Total RNA or mRNA from human fibroblast TIG-1 cells was prepared as described previously.[7] Ex*Taq* DNA polymerase for RT–PCR was purchased from TaKaRa Co. Ltd. (Otsu, Japan). Probe labeling and detection for northern blots were performed by using the Gene Images Random-Prime Labelling and Detection System (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

2.3. *Preparation of the subtracted cDNA library and stepwise subtraction*

Poly(A)$^+$ RNA was purified from total RNA by oligo(dT) cellulose chromatography. A cDNA library

with eight million independent clones was constructed from the PBMC mRNAs by using the linker-primer method and the pAP3neo vector as described previously.[7] The poly(A)$^+$ RNAs from exponentially growing TIG-1 cells that had been incubated with 10% serum in tissue culture plates were also purified and biotinylated by using photobiotin. After converting the cDNA library to a single-stranded form by transfection with an f1 helper phage, we hybridized it with the biotinylated mRNAs and subtracted it by biotin–avidin interactions.[7] The unhybridized clones were converted to the double-stranded form and used to transform competent *Escherichia coli* cells. This generated a first-stage subtracted cDNA library of 11 million independent clones.

We then prepared plasmid DNA from ~800 randomly selected cDNA clones and numbered and digested an aliquot of each plasmid DNA with *Sma*I and *Not*I restriction enzymes to prepare 10 sheets of Southern blots, each of which included 80 clones arranged in order. We then purified the cDNA inserts of clones 1–20 on 1% agarose gels by digesting them with *Eco*RI and *Not*I; these inserts were labeled with fluorescent dye and then used as probes for northern analysis with PBMC and TIG-1 RNAs to determine which clones contained *PREP* genes (data not shown). The DNA sequences of the *PREP* clones from the 5′ end of the cDNA inserts were determined by the dideoxy-chain termination reaction using an automatic DNA sequencer (Licor 4000L; Lincoln, NE). After these analyses, we selected the next 20 unhybridized clones on the Southern blot (from 21 onwards) for the next round of cDNA insert preparation, fluorescent labeling, and northern analysis. This procedure was repeated until we finished testing all 800 unhybridized clones. The *PREP* genes identified in the preceding step were then converted to RNA, biotinylated with photobiotin, and used to subtract the first-stage subtracted cDNA library. The second-stage subtracted cDNA library was then analyzed as described above and subtracted again. This process was repeated three times as described previously.[4]

After the clones whose transcription was conspicuously up-regulated in PBMCs as compared with TIG-1 cells were sequenced, the DNA sequences were used to search the EST database by using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4. *Human cDNA microarray analysis for identification of PREP genes*

The total RNAs (500 ng) from the normal human PBMCs were pooled, as were the TIG-1 cell RNAs, after which both pools were reverse-transcribed by using oligo-dT primers containing the T7 RNA polymerase promoter sequence. The cDNAs were then subjected to *in vitro* transcription using T7 RNA polymerase to label the complementary RNAs (cRNAs) with cyanine 3 (Cy3)-CTP or cyanine 5 (Cy5)-CTP (Amersham Pharmacia Biotech, Piscataway, NJ). The Cy-labeled cRNAs from the normal human PBMCs (1 μg) were then mixed with the same amount of reverse color Cy-labeled TIG-1 cell-derived cRNAs. Hybridizations, rinsing, scanning, and gene analysis on the Agilent's all human cDNA microarray (Hu44K) were conducted according to the manufacturer's protocol (G2940BA; Agilent Technologies, Inc., Palo Alto, CA). Fluorophore reversal (dye swap) duplicates were used in two-color DNA microarray experiments. The 399 genes that showed the highest level of up- or down-regulation were selected and subjected to RT–PCR analysis using the normal human PBMC and TIG-1 RNAs (Fig. 1) with relevant oligonucleotide primers (Supplementary Table S2). Of these, 122 genes were identified as *PREP* genes, 33 of which were already identified as *PREP* genes by stepwise subtraction.[4]

2.5. *Individual microarray analysis on CSS patients*

The quality of the RNA samples obtained from PBMCs of each CSS patient was examined by using the RNA 6000 Nano LabChip Kit (p/n 5065-4476) on the Agilent 2100 Bioanalyzer. To conduct the individual cDNA microarray (Hu44K) analysis on CSS patients and normal volunteers, we generated fluorescently labeled cRNA by *in vitro* transcription with T7 RNA polymerase in the presence of Cy5-CTP or Cy3-CTP using a Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, Inc.) as described above. The Cy5-labled cRNA of each patient was then mixed with the Cy3-labled cRNA of normal volunteers to probe the cDNA microarray. *PREP* gene expression analysis was then conducted by using the Gene Spring software 7.3.1 (Agilent Technologies, Inc.) by setting appropriate parameters to select 33 or 3 *PREP* genes whose expressions are up-regulated (>1.2-fold change) or down-regulated (<1.0-fold change) in all seven CSS patients as compared with healthy volunteers. We also conducted similar analysis to select nine conspicuously up-regulated *PREP* genes (>2.0-fold change).

3. Results

3.1. *Isolation of putative PBMC-specific genes*

To isolate putative human PBMC-specific genes, we first prepared mRNAs from the PBMCs of healthy volunteers and generated a human PBMC cDNA library with 80 million independent clones by using the linker-primer method described previously.[7] We also prepared mRNAs from exponentially growing normal