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Neutrophil activation and arteritis induced by *C. albicans* water-soluble mannoprotein-β-glucan complex (CAWS)

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Abstract

We have established a mouse model which shows the symptoms of coronary arteritis after consecutive injections of CAWS, which is released from *Candida albicans*. In this study, we examined neutrophil activation in the initial period after CAWS injection intraperitoneally. During 10 min to 16 h after the injection, blood profiles and neutrophil functions were determined. At the same time, levels of inflammatory cytokines and chemokines in plasma were measured. Furthermore, level of ICAM-1 as a marker of lesion in arterial endothelial cells was measured. Counts of the peripheral leukocytes increased immediately after CAWS injection, especially involving neutrophil. In vitro sensitivity of neutrophils to stimuli was enhanced. Moreover, proinflammatory cytokines (IL-1β, IL-12 and IL-6) increased in plasma initially followed by an increase in IL-10, G-CSF, MIP-2 and soluble ICAM-1. Locally, ICAM-1 message in arterial walls was significantly increased 16 h after CAWS injection. A decrease in C3 levels was observed in plasma, suggesting complement activation and consumption. In summary, neutrophil activation occurred after CAWS injection, followed by complement activation, and production of proinflammatory cytokines chemokines and G-CSF which may be involved in development of coronary arteritis.

Keywords: Complement activation; Inflammatory cytokines; Endothelium injury

Introduction

Recently, clinical relevance of fungal diseases has increased, mainly because of an increasing population of immunocompromised individuals including those infected with HIV, transplant recipients and patients with cancer (Zupanic-Krmek and Nemet, 2004). Neutrophils play a key role during *Candida albicans* (*C. albicans*) infection (Urban et al., 2006; Fradin et al., 2005). *C. albicans* organisms exposed to erythrocytes, mononuclear cells, plasma or blood lacking neutrophils become physiologically active and rapidly switch to filamentous growth. In contrast, the presence of neutrophils arrested *C. albicans* growth (Urban et al., 2006; Fradin et al., 2005). Recently, immune responses to some components of *C. albicans* such as mannan (Muller and Melchinger, 2004) or β-glucan (Gantner et al., 2005) have been reported.

Abbreviations: CAWS, *C. albicans* water-soluble mannoprotein-β-glucan complex; fMLP, fMet-Leu-Phe.

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Animal model is useful to clarify mechanism of infection process, genetic background differences and for development of a strategy for therapy (Nagi-Miura et al., 2004; Aratani et al., 2002; Spellberg et al., 2005).

Some microorganisms or microorganism-derived products are associated with specific diseases. For example, certain *Campylobacter jejuni* strains trigger Guillain–Barre syndrome while other strains trigger the Fisher syndrome in the Japanese population (Takahashi et al., 2005). Esper et al. have reported that New Haven coronavirus (HCoV-NH) infection is associated with Kawasaki disease (Esper et al., 2005). Recently, Iwai et al. have identified oral microorganisms in the lesions of Buerger disease, suggesting a possible etiologic link between Buerger disease and chronic infections such as oral bacterial infections (Iwai et al., 2005). We have established a mouse model which shows the symptoms of coronary arteritis following consecutive injections into mice of a water-soluble polysaccharide from *Candida* sp. (CAWS) (Nagi-Miura et al., 2004; Ohno, 2003). In this study, we examined neutrophil activation and subsequent events after a single injection of CAWS (Fig. 1).

Materials and methods

Reagents

ELISA kits for mouse IL-1β, IL-6, IL-10, IL-12 p70, IFN-γ, TNF-α were purchased from BD Biosciences (CA, USA), IL-18 was from Medical and Biological Laboratories (Nagoya, Japan), soluble ICAM-1 and MIP-2 were from R&D Systems (MN, USA), G-CSF and GM-CSF were from AN'ALYZA (MN, US). fMet-Leu-Phe (fMLP) was purchased from Peptide Institute (Osaka, Japan), 3,3′, 5,5′-tetrametylbendizine (TMB), cytochalasin B (CB), cytochrome *c*, RPMI 1640 medium, aprotinin and PMSF were purchased from Sigma Chemical Co. (MO, USA). Casein was purchased from Calbiochem. Co. (Darmstadt, Germany). TaqMan Universal PCR master mix was purchased from PE Biosystems (NJ, USA). Monoclonal antibody and peroxidase-conjugated IgG fraction to mouse C3 were purchased from HyCult biotechnology (Uden, The Netherlands) and MP Biochemicals, Inc. (CA, USA), respectively.

Mice

C57BL/6N male mice were purchased from Charles River Japan, kept under SPF conditions, and used according to a guideline for animal care of the National Institute of Infectious Diseases. The mice were used in the experiment at 6 weeks of age.

Preparation and administration of CAWS

CAWS was prepared from *C. albicans* strain IFO1385 in accordance with conventional methods (Nagi-Miura et al., 2004). CAWS was dissolved in PBS. After autoclaving, 0.2 ml of the material (20 mg/ml) was intraperitoneally injected into a mouse. For vitro assay, neutrophils isolated from 8% casein-induced peritoneal exudated cells were co-cultured with CAWS in RPMI medium containing 0.3 mM PMSF and 0.4 μg/ml aprotinin.

Histological observations of coronary arteritis

Coronary artery segments were fixed in 10% buffered (pH7.2) formaldehyde, paraffin-embedded, and sections were stained with hematoxylin and eosin.

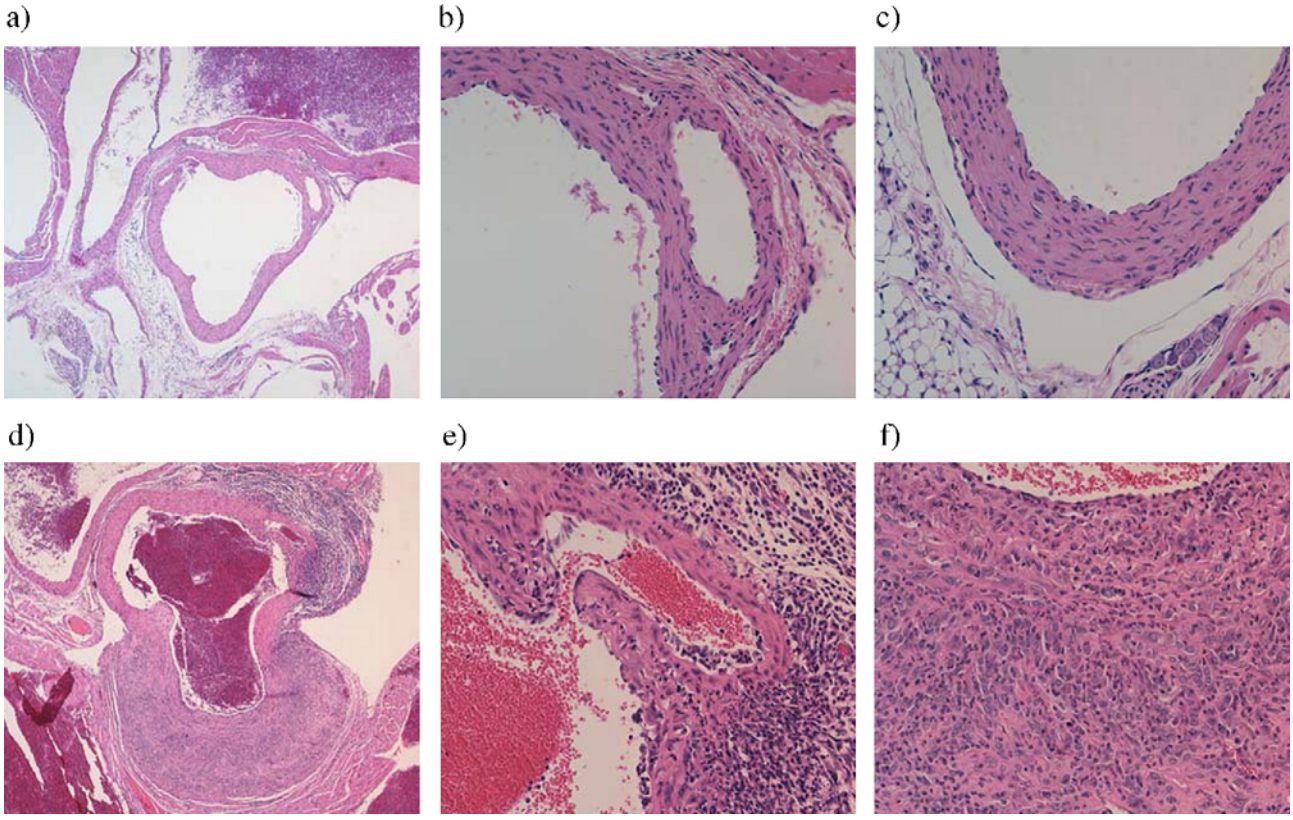

Fig. 1. Histological analysis of coronary arteritis administration of a single dose of CAWS. Normal (a) aortic root including coronary artery ×40, (b) coronary artery ×200 and (c) aorta, ×200. Histology 4 weeks after CAWS injection a single dose of 4 mg/mouse (d) vasculitis at the coronary artery and aortic root, ×40, (e) coronary arteritis, ×200, (f) aortitis, ×200.

Analysis of leukocytes profile

Peripheral blood was collected from heart and EDTA-2K was added. Leukocyte profiles were analyzed by use of automation instruments (Mitsubishi BCL Corp., Tokyo, Japan).

Flow cytometric analysis

Peripheral blood was collected from heart and EDTA-2K was added. After lysis for 5 min at 4 °C, subsequent centrifugation (1000×*g* at 4 °C, cells) was suspended in HBSS. Bone marrow cells were prepared from a shinbone. The cells were incubated with mAb against surface markers (BD Pharmingen, CA, USA). Gr-1 (RB6-8C5), Mac-1 (M1/70) were used for flow cytometric analysis, and then analyzed with FACSCalibur using CellQuest software (BD Biosciences, CA, USA).

Neutrophil function assay

Heparinized blood was collected from heart, and neutrophil was prepared. MPO release and superoxide generation were measured as described previously (Ishida-Okawara et al., 1991).

Measurement of proinflammatory cytokines and chemokine in plasma

Levels of proinflammatory cytokines IL-1β, IL-6, IL-10, IL-12, IL-18, TNF-α, INF-γ, MIP-2, soluble ICAM-1, G-CSF, GM-CSF in plasma using individual ELISA.

Preparation of peritoneal exudate neutrophils and measurement of cytokine production level by co-cultured neutrophil with CAWS

Normal C57BL/6N mice were intraperitoneally injected with 4 ml of 8% casein in PBS and exudate cells containing resident macrophage or casein-induced neutrophils were recovered 8 h lavage with 5 ml of PBS. The exudate cells were put onto M-SMF (Japan Immunoresearch Laboratories Co., Ltd., Takasaki, Japan), centrifuged 1200×*g* for 20 min at room temperature. Neutrophils were suspended (5×10^6 cells/ml) in RPMI-1640 medium containing 0.3 mM PMSF and 0.4 μg/ml aprotinin and co-cultured with 1 mg/ml CAWS for 0.5 to 4 h. At the end of culture, culture supernatant was prepared by centrifugation and levels of cytokines IL-1β, IL-6, IL-10 were determined using by ELISA kits.

Measurement of ICAM-1 mRNA expressed in aortic wall after CAWS injection

The thoracic aortas of mice were isolated and frozen immediately for detection of ICAM-1 mRNA. The total aortic RNA for each mouse was isolated using ISOGEN (Nippon ene, Tokyo Japan). One microgram of RNA was reverse transcribed with ReverTra Ace (Toyobo, Osaka, Japan) to obtain cDNA. Real-time PCR was performed using ABI PRISM 7000 Sequence Detection System (Applied Biosystems, CA, US) according to the manufacture's protocol. Primers and TaqMan probes specific for ICAM-1 was obtained from Assay-on-Demand Gene Expression Products (Applied Biosystems). For endogenous control, the level of GAPDH in each sample was measured using TaqMan Rodent GAPDH Control Reagents VIC (Applied Biosystems). Data analyses were performed on ABI PRISM 7000 SDS software version 1.0 (Applied Biosystems).

Detection of mouse complement 3 (C3) by ELISA and Western blotting

Peripheral blood was collected from heart and EDTA-2K was added. One μg/ml of C3 monoclonal antibody was coated to ELISA plate for overnight. After washing, blocking was done by 50% FCS for 1 h. Sample plasma (×100) was added to the plate for 1 h, after washing peroxidase labeled-2nd antibody (×1000) was added to the plate. After washing, *o*-phenylenediamine chloride was added to the plate for 3 min. Finally, reaction was stopped 2 N hydrogen sulphate and measured at 490 nm by auto reader (Nippon Bunko, Tokyo, Japan). Western blotting was performed by using mouse C3 monoclonal antibody and peroxidase-labeled anti-Rat IgG as 2nd antibody.

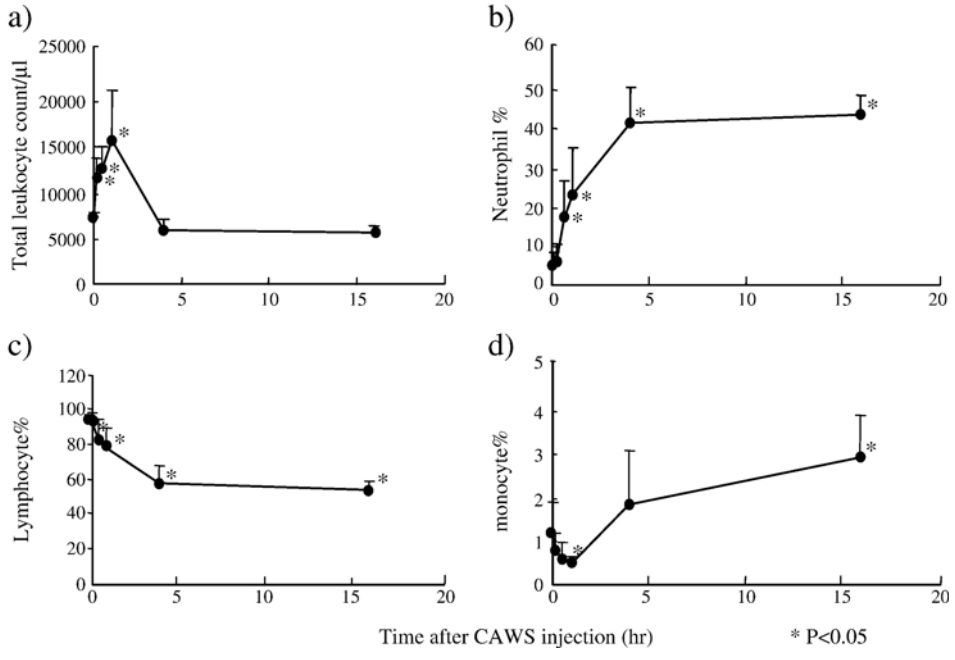

Fig. 2. Change of blood profile after CAWS injection. Peripheral blood was collected from heart in EDTA-2K. Blood profile was analyzed by Mitsubishi Kagaku BCL Inc. (a) total leukocyte number, ratio of (b) neutrophil, (c) lymphocyte and (d) monocyte, respectively. $N=6$ in each group.

Table 1
Change of Gr-1$^+$ cells ratio after CAWS injection

	Time after CAWS injection (hr)					
	0	0.17	0.5	1	4	16
Bone marrow	19.47±5.35	15.85±1.89	9.65±1.14*	10.42±1.26*	7.39±1.97*	11.73±6.58
Peripheral blood	5.24±6.92	5.30±0.36	10.35±2.00*	9.78±3.59	8.06±6.69	6.12±5.34

* $P<0.05$.

Statistical analysis

Results expressed as the mean±SD. Statistical analysis was using Mann–Whitney *U*-test. The probability value of <0.05 was considered significant.

Results

Observation of coronary arteritis administration of a single dose of CAWS

We have severe coronary arteritis by daily injection of CAWS (4 mg/mouse/day) for 5 days. In order to compare initial events and development of coronary arteritis, the current studies employed a one shot injection of CAWS at a dose of 4 mg/mouse. Four weeks after CAWS injection with this protocol, arteritis was evident in both coronary and aorta (Fig. 1).

Profiles of peripheral leukocytes

After CAWS injection, total leukocyte counts were increased and later returned to baseline levels 4 h (Fig. 2a). The ratio of lymphocytes decreased after CAWS injection, while ratio of neutrophils promptly increased (Figs. 2b, c), the ratio of monocyte increased (Fig. 2d).

Profile of leukocyte by flow cytometric analysis

To confirm the leukocyte counts, flow cytometric analysis of peripheral and bone marrow cells after CAWS injection was done (Table 1). Bone marrow cell numbers the tibia were significantly increased 4 h after CAWS injection (data not shown). The ratio of Gr-1 cells in bone marrow significantly decreased at 0.5, 1, 4 h after CAWS injection, while the ratio of the cells in peripheral blood increased suggesting that neutrophils in bone marrow promptly migrate to peripheral blood after CAWS injection.

CAWS effect on neutrophil activation

To assess the functional response of neutrophils after CAWS injection, MPO release and superoxide generation were

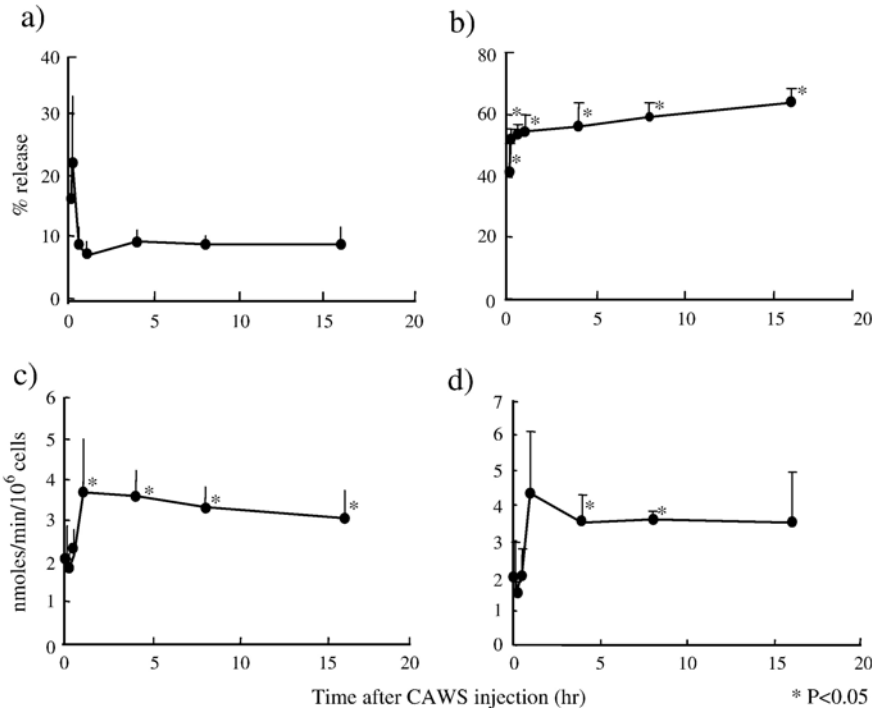

Fig. 3. Neutrophil activation after CAWS injection. (a, b) MPO release from neutrophils after CAWS injection. Neutrophils (10^6 cells/ml) were stimulated in the (a) absence, or (b) presence of fMLP (10^{-5} M) and cytochalasin B (5 μg/ml). (c, d) Superoxide generation after CAWS injection. Neutrophils (10^6 cells/ml) were stimulated with (c) fMLP (10^{-5} M) and cytochalasin B (5 μg/ml) or (d) PMA (1 μg/ml). $N=6$ in each group.

examined (Fig. 3). fMLP-induced MPO release from neutrophils was enhanced after CAWS injection (Fig. 3b), while spontaneous MPO release was not changed (Fig. 3a). In addition, both fMLP and PMA-induced superoxide generation was enhanced following in vivo CAWS injection (Figs. 3c, d).

CAWS effects on proinflammatory cytokine production

Since activation of neutrophils was observed, after in vivo injection of CAWS, proinflammatory cytokines levels in plasma were measured (Fig. 4). IL-12 p70 production was increased (Fig. 4d), while levels of IL-1β (b), IL-10 (c), 1L-6 (d) were significantly increased transiently in the first 1–2 h after CAWS injection. Levels of MIP-2 and G-CSF in plasma increased several hours after CAWS injection and were maintained 16 h after CAWS injection (Figs. 4e, f). On the other hand, IL-18, TNF-α, INF-γ, GM-CSF were not detected up to 16 h after CAWS injection (data not shown). Since IL-1β, IL-10, IL-6 was significantly increased, production of these cytokines by casein-induced neutrophils was also measured (Fig. 5). IL-6 production was enhanced by exposure of neutrophils to CAWS (Fig. 5c), while those of IL-1β and IL-10 were nearly the same in presence or absence of CAWS (Figs. 5a, b).

CAWS effect on ICAM-1 expression and soluble ICAM release

Since ICAM-1 is a marker of activation of endothelial cells, we assayed plasma for soluble ICAM-1 (sICAM-1) after in vivo infusion of CAWS. sICAM-1 gradually increased in plasma after CAWS injection (Fig. 6a). In addition, ICAM-1 mRNA in extracts from the thoracic aortic wall was also significantly increased 16 h after CAWS injection (Fig. 6b).

Complement activation by CAWS

Activation of complement was examined (Fig. 7). C3 in plasma decreased time dependently after CAWS injection and gradually returned to normality thereafter (Fig. 7a). This was confirmed by Western blotting analysis (Fig. 7b). These results suggest that neutrophil activation triggered through complement activation by a single injection.

Discussion

In study, we focused on neutrophil activation related to development of coronary arteritis. Single injection of CAWS at a dose of 4 mg/mouse induced coronary arteritis 4 weeks, featuring intense neutrophil accumulation in the coronary arterial wall. This observation was similar

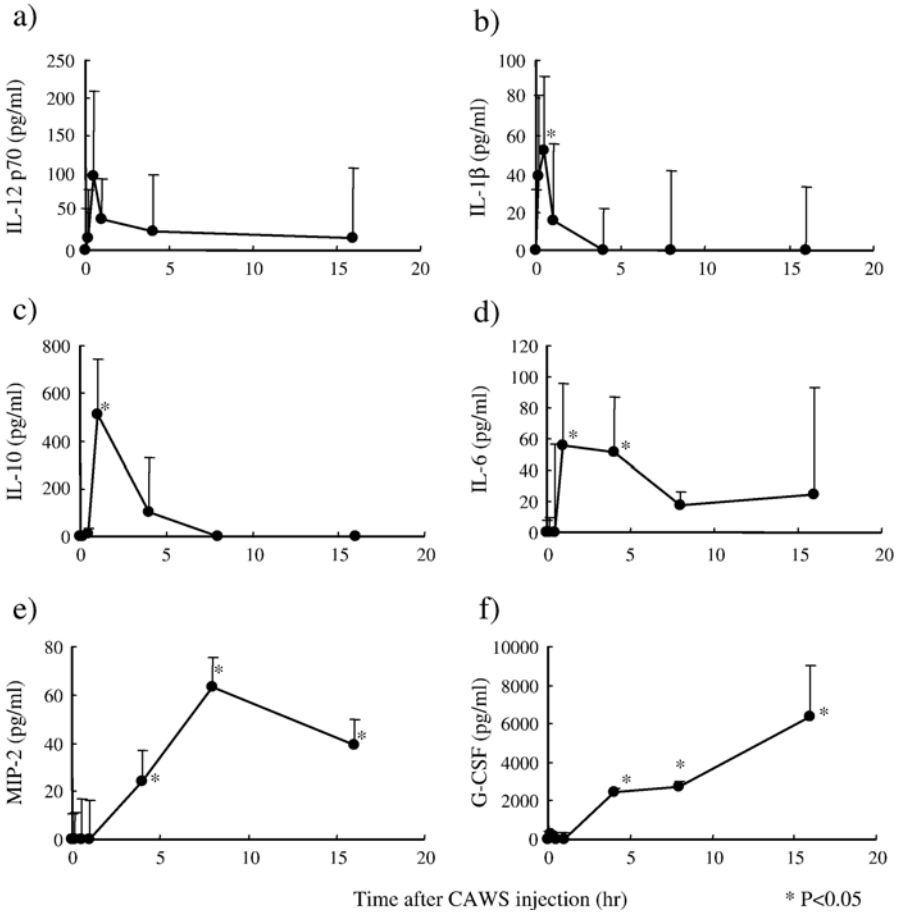

Fig. 4. Cytokine release into plasma after CAWS injection. Heparinized blood was obtained from heart after CAWS injection. Plasma was separated and proinflammatory cytokines level was measured by ELISA kit. (a) IL-12 p70, (b) IL-1β, (c) IL-10, (d) IL-6, (e) MIP-2, (f) G-CSF, respectively. $N=6$ in each group.

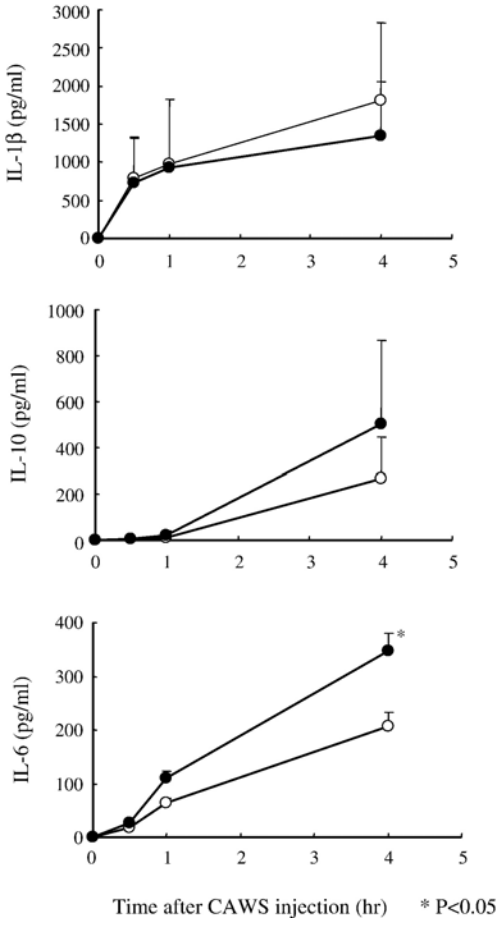

Fig. 5. Cytokine production by neutrophil. Casein-induced neutrophil (5×10^6 cells/ml) was suspended in RPMI-1640 containing 0.3 mM PMSF and 1.4 μg/ml aprotinin and co-cultured with 1 mg/ml CAWS for 0.5 to 4 h. The culture supernatant was prepared by centrifugation and level of cytokine. (a) IL-1β, (b) IL-10 and (c) IL-6, was measured respectively. ○ not treated by CAWS ● 1 mg/ml of CAWS. N=3–6 in each group.

to the development of coronary arteritis daily injection of CAWS (4 mg/mouse/day) 5 days (Nagi-Miura et al., 2004; Ohno, 2003).

Blood neutrophils and subsequently monocyte increased, while lymphocyte counts decreased. In addition, the ratio of Gr-1 cells in bone marrow decreased, suggesting migration of neutrophils from bone marrow into peripheral blood after CAWS injection. G-CSF after CAWS injection, fMLP-induced MPO release from neutrophils was enhanced as was PMA and fMLP-induced superoxide generation in vivo injection. These results suggest that CAWS increases the number of blood neutrophils and induces their activation early after CAWS injection.

Levels of IL-1β, IL-6, and 1L-10 significantly increased in plasma after CAWS injection. These cytokines are also released from blood leukocytes live *C. albicans* (Netea et al., 2002; Gasparoto et al., 2004). The type of cytokines appearing seems to be different depend on *Candida* strain, component and virulence (Villar et al., 2005). With regard to casein-induced neutrophils, IL-6 was significantly enhanced by co-culture with CAWS, but IL-1β and 1L-10 production virtually unchanged in presence of CAWS. These results suggest IL-6 production was especially enhanced by CAWS. The levels of MIP-2 and G-CSF in plasma were maintained for 16 h, neutrophil levels in blood, MIP-2 and G-CSF in blood.

The cause of increases and activation of peripheral neutrophils after CAWS injection is not known. Complement activation products may well be candidates because C5a is a chemoattractant for and activator of neutrophils (Guo and Ward, 2005). Ohno et al. have already demonstrated activation of the lectin complement pathway by CAWS (unpublished). Mullick A. et al. have confirmed dysregulated cytokine response during *C. albicans* infection in $C5^{-/-}$(Mullick et al., 2004).

Soluble ICAM-1, which is a marker of activated endothelial cells (Collins et al., 2000; Iiyama et al., 1999), in blood increased after CAWS injection. In addition, ICAM-1 message was significantly increased in the thoracic aortic wall 16 h after CAWS injection. Both systemic and local increases in ICAM-1 could be involved to subsequent endothelial cell lesion development (Di Lorenzo et al., 2004).

In summary, increased numbers and activation of peripheral blood neutrophils are the initial events after CAWS injection,

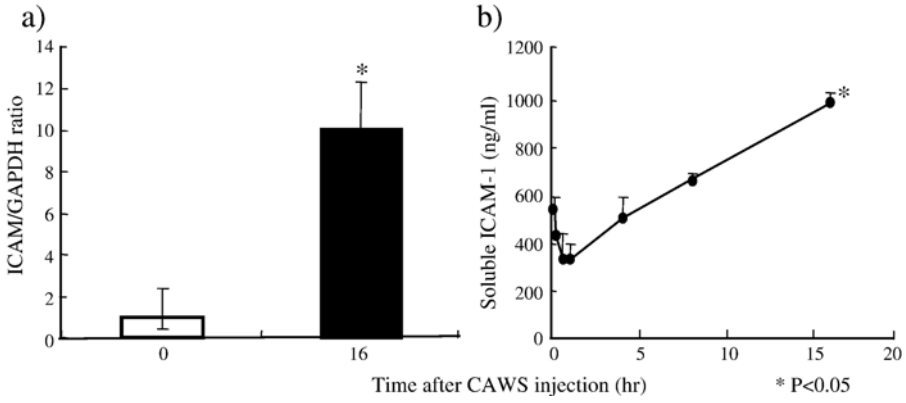

Fig. 6. Soluble ICAM-1 in plasma and ICAM-1 mRNA in aorta after CAWS injection. (a) Heparinized blood was obtained from heart after CAWS injection. Plasma was separated and ICAM-1level was measured by ELISA kit. (b) Total RNA was extracted and mRNA was isolated. cDNA was prepared from 1 μg of mRNA. Real-time PCR was performed and analyzed. GAPDH was used as an internal control. White bar shows not treated by CAWS, black bar shows treated by 4 mg/mouse of CAWS, respectively. N=6 in each group.

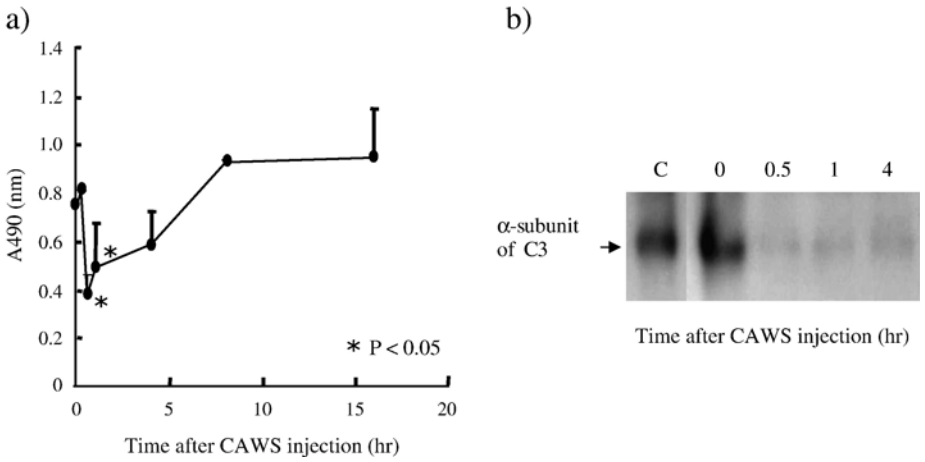

Fig. 7. Mouse complement 3 (C3) was assessed in plasma by sandwich ELISA and Western blotting. 10 mM EDTA-2K treated blood was prepared from heart after CAWS injection. (a) C3 protein was detected by sandwich ELISA using monoclonal and peroxidase-labeled polyclonal antibody, (b) α-chain of C3 was detected by monoclonal antibody to mouse C3.

perhaps followed by macrophage activation and adaptive immune responses. It is believed that neutrophils have a primary role in host defense against *C. albicans* or its products. Arteritis induced by CAWS might be related to activation of neutrophils in circulated blood and their accumulation in arterial and aortic walls where ICAM-1 has been expressed.

Acknowledgments

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