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Original Article

JNK is critical for the development of *Candida albicans*-induced vascular lesions in a mouse model of Kawasaki Disease

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ABSTRACT

Background: Kawasaki disease (KD) is the most common systemic vasculitis of unknown etiology in children, and can cause the life-threatening complication of coronary artery aneurysm. Although a novel treatment strategy for patients with KD-caused vascular lesions is eagerly awaited, their molecular pathogenesis remains largely unknown. c-Jun N-terminal kinase (JNK) is a signaling molecule known to have roles in inflammation and tissue remodeling. The aim of this study was to elucidate significant involvement of JNK in the development of vascular lesions in a mouse model of KD.

Methods and results: We injected *Candida albicans* cell wall extract (CAWE) into 4-week-old C57BL/6 mice. Macroscopically, we found that CAWE caused the development of bulging lesions at coronary artery, carotid artery, celiac artery, iliac artery and abdominal aorta. Histological examination of coronary artery and abdominal aorta in CAWE-treated mice showed marked inflammatory cell infiltration, destruction of elastic lamellae, loss of medial smooth muscle cells and intimal thickening, which are similar to histological features of vascular lesions of patients with KD. To find the role of JNK in lesion formation, we evaluated the effects of JNK inhibitor, SP600125, on abdominal aortic lesions induced by CAWE. Interestingly, treatment with SP600125 significantly decreased the incidence of lesions and also protected against vascular inflammation and tissue destruction histologically, compared with the placebo treatment.

Conclusions: Our findings suggest that JNK is crucial for the development of CAWE-induced vascular lesions in mice, and potentially represents a novel therapeutic target for KD.

1. Introduction

Kawasaki disease (KD), which is the most common systemic vasculitis with unknown origin in childhood, causes inflammation of coronary arteries, leading to development of aneurysms, a life-threatening complication [1–4]. About 25% of children with untreated KD and 5% of those treated with high-dose γ-globulin, the current standard therapy for acute-stage KD [3,5], develop coronary artery aneurysms and remain at risk of myocardial infarction and sudden death until later in life [3,6,7]. Therefore, the need for novel therapeutic targets in KD-caused vascular lesions is critical. However, the pathogenesis of KD is not fully understood.

Some experimental studies of KD that used mouse models have provided potential therapeutic targets for coronary artery vasculitis. In these studies, *Lactobacillus casei* cell wall extract (LCWE) and *Candida albicans* cell wall extract (CAWE) were commonly used to induce a mouse model of coronary arteritis that mimics that of human KD [8–11]. For example, Lee et al. demonstrated the crucial role of interleukin-1β in LCWE-induced coronary arteritis in mice [10]. Martinez et al. reported the role of chemokine receptor-2 in CAWE-induced coronary arteritis in mice [12]. Oharaseki et al. also used CAWE-induced coronary arteritis in mice to examine the role of tumor necrosis factor-α [11]. Although extensive histopathological analyses were conducted to evaluate inflammation in these studies, we needed to

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establish a new experimental model system, to enable both macroscopic and histopathologic evaluation of KD-caused vascular lesions.

All aneurysmal lesions, whatever their etiology, share common pathologic hallmarks, including inflammation and proteolytic degradation of the extracellular matrix [13–16]. Excessive matrix proteolysis mediated by matrix metalloproteinases (MMPs), notably MMP-9, is considered a common and critical step during lesion development [13,15,17,18]. In fact, MMP-9 is upregulated in coronary lesions of the LCWE-induced mouse model [19] and also in patients with KD [20]. Inhibition of MMP-9 had been shown to prevent elastin degradation in the LCWE-induced mouse model, but it had no effect on inflammatory infiltration [19], which suggests that upstream signaling molecules would be a desirable target. We then focused on c-Jun N-terminal kinase (JNK), a stress-activated signaling molecule, which regulates MMP-9 and various proinflammatory cytokines [21,22]. SP600125, a specific JNK inhibitor, has been shown to completely block development of abdominal aortic aneurysm in mice, accompanied by reduction of MMP-9 and macrophage infiltration, and preservation of elastic lamellae [23].

We hypothesized that inhibiting JNK would attenuate development of vascular lesions in a mouse model of KD. Initially, we successfully created a mouse model system that allowed us to assess development of the lesions that are compatible with those in KD. Consequently, we showed that pharmacologic inhibition of JNK effectively prevented development of CAWE-induced lesions in mice.

2. Methods

2.1. CAWE preparation

Candida albicans cell wall extract (CAWE) was prepared from *Candida albicans* standard strain SC5314 by modifying the method described previously [9]. Briefly, *Candida albicans* SC5314 stock culture was stored at −80 °C, then incubated at 37 °C for 48 h aerobically on yeast peptone dextrose agar (10 g yeast extract, 20 g peptone, 20 g glucose and 20 g agar per liter). Yeast cells were harvested (about 600 mg wet weight/plate) from agar plates using a scraper and washed three times with distilled water. An extract was obtained by boiling yeast cells for 2 h with 0.5 M KOH (200 mg wet weight of yeast cells/ml). After alkali neutralization in pH7.2 and dialysis against water for 3 days, the extracted material was precipitated with ethanol. The precipitate, about 4% against wet weight yeast cells, was suspended in phosphate-buffered saline (PBS) and adjusted to a final concentration of 100 mg/ml.

2.2. Mice

Four-week-old C57BL/6 N male mice were purchased from Kyudo Co., Ltd. (Tosu, Saga, Japan). Mice were kept in plastic cages (5 per cage) under pathogen-free conditions in a room at 24±2.5 °C and 55%±5% relative humidity under a 12:12-h light–dark cycle. Mice were given free access to standard food and water throughout the experiments. All experiments were performed in conformity with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. The protocols were approved by the Laboratory Animal Care and Use Committee of Fukuoka University (#116479).

2.3. Induction of vascular lesions in mice

To induce vascular lesions, 4-week-old C57BL/6 male mice were injected intraperitoneally (i.p.) with 4 mg of CAWE for 5 consecutive days every 4 weeks for 2 cycles; and then euthanized with overdoses of sodium pentobarbital (100 mg/kg, i.p.) at 4, 8 or 12 weeks after the second CAWE cycle (Fig. 1A). For whole-body perfusion fixation, 4% paraformaldehyde in PBS was perfused at physiological pressure. After perfusion fixation, the hearts and the whole aortas with branches were exposed and excised for morphometric and histological analyses. Additionally, in some experiments, a mixture of 10% India ink/4% gelatin in PBS was injected into aortic root to visualize coronary arteries.

2.4. Inhibition of JNK in mice

Custom-made pellets containing JNK-specific inhibitor SP600125 (30 mg/kg/day) and control placebo pellets were purchased from Innovative Research of America (Sarasota, FL, USA). For pellet implantation, 4-week-old C57BL/6 male mice were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). Anesthesia was monitored by periodic observation of respiration and pain response. Pellets were implanted in subcutaneous pockets created on the backs of the mice before starting CAWE administration as described above. The mice were euthanized as described, at 4 weeks after the second CAWE cycle (Fig. 1B). After whole-body perfusion fixation as described, hearts and whole aortas with branches were excised, photographed for morphometric analysis, and analyzed histologically. Photographs of aortas were used to determine maximum external aortic diameters.

2.5. Histological and immunohistochemical analyses

Paraffin-embedded sections were stained with hematoxylin/eosin (HE) and elastica-van Gieson (EVG) for histological analysis. For EVG staining, sirius red was used instead of acid fuchsin. Sections were also probed with antibodies raised against appropriate antigens for immunohistochemistry, as described previously [24]. We detected tenascin-C (TN-C), α-smooth muscle actin (α-SMA), Mac-3 and activated JNK by probing sections with rabbit polyclonal anti-TN-C antibody [25], mouse anti-smooth muscle α-actin antibody (Dako, Glostrup, Denmark), rat anti-Mac-3 antibody (BD Biosciences, San Jose, CA, USA) and rabbit polyclonal anti-phosphorylated JNK (p-JNK) antibody (Promega, Fitchburg, WI, USA), respectively. The sections were visualized with an avidin–biotin–peroxidase complex staining kit (Vector Laboratories, Burlingame, CA, USA) and colorized with diaminobenzidine (DAB) chromogen. For double immunostaining, sections were incubated with anti-p-JNK antibody, visualized with a peroxidase complex staining kit and DAB, and incubated with fluorescein isothiocyanate-conjugated anti-α-SMA (Sigma, St. Louis, MO, USA) or anti-Mac-3 and Alexa Fluor 546 goat anti-rat IgG (Molecular Probes, Eugene, OR, USA). Slides were observed under a fluorescent/differential interference contrast (DIC) microscope (BH2, Olympus, Tokyo Japan). Immunofulorescent signals were superimposed on DIC images.

2.6. Statistical analysis

Data are expressed as mean±standard deviation (SD). Statistical analyses were performed with the Prism 5.0d statistical program (GraphPad Software, La Jolla, CA, USA). We used Fisher's exact test to compare incidence of aneurysm development. We used the Mann Whitney test to compare maximal aortic diameters between experimental groups. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Development of arterial and aortic lesions induced by CAWE

To create a mouse model of KD-related lesions, we injected into 4-week-old C57BL/6 male mice with 4 mg of CAWE for 5 consecutive days for 2 cycles. Four to 12 weeks later, we found that a considerable number of mice developed bulging lesions and that these lesions were created at abdominal aorta, iliac artery, coronary artery, carotid artery, and celiac artery (Fig. 2A-D). Some mice had multiple lesions with a "string of beads" appearance (Fig. 2D). All of the lesions looked pearly white, which indicated that they were