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Critical role of caspase-1 in vascular inflammation and development of atherosclerosis in Western diet-fed apolipoprotein E-deficient mice

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ABSTRACT

Objective: Recent investigations have suggested that the inflammasome plays a role in the development of vascular inflammation and atherosclerosis; however, its precise role remains controversial. We produced double-deficient mice for apolipoprotien E (*Apoe*) and caspase-1 (*Casp1*), a key component molecule of the inflammasome, and investigated the effect of caspase-1 deficiency on vascular inflammation and atherosclerosis.

Methods and results: Atherosclerotic plaque areas in whole aortas and aortic root of Western diet (WD)-fed $Apoe^{-/-}Casp1^{-/-}$ mice were significantly reduced compared to those in $Apoe^{-/-}$ mice. The amount of macrophages and vascular smooth muscle cells in the plaques was also reduced in $Apoe^{-/-}Casp1^{-/-}$ mice. No significant differences in plasma lipid profiles and body weight change were observed between these mice. Expression of interleukin (IL)-1β in the plaques as well as plasma levels of IL-1β, IL-1α, IL-6, CCL2, and TNF-α, in $Apoe^{-/-}Casp1^{-/-}$ mice were lower than those in $Apoe^{-/-}$ mice. *In vitro* experiments showed that calcium phosphate crystals induced caspase-1 activation and secretion of IL-1β and IL-1α in macrophages.

Conclusion: Our findings suggest that caspase-1 plays a critical role in vascular inflammation and atherosclerosis, and that modulation of caspase-1 could be a potential target for prevention and treatment of atherosclerosis.

1. Introduction

Atherosclerosis is a chronic inflammatory disease characterized by lipid deposition, leukocyte infiltration, and vascular smooth muscle cell (VSMC) proliferation in the vascular walls [1]. The inflammatory nature of atherosclerosis is evidenced by many findings. For instance, the inflammatory cells, mainly macrophages, infiltrate into the atherosclerotic plaques and the number of these cells is linked to the severity of the disease. Inflammatory cytokines and chemokines are involved in all stages of the process of atherosclerosis [2]. Furthermore, the association between cardiovascular events and serum inflammatory markers, particularly C-reactive protein, has been demonstrated [3]. However, the molecular basis by which an inflammatory response can occur in the process of atherosclerosis is not known.

Inflammation in the atherosclerotic process is considered sterile inflammation because it mostly occurs in the absence of microbial infection [4]. A number of inflammatory cytokines are involved in the inflammatory response of atherosclerosis. Of these, clinical and experimental studies have shown the importance of IL-1β in the pathogenesis of atherosclerosis [2,5]. A growing body of evidence suggests that some types of sterile inflammation are mediated through a newly discovered innate immune pathway known as the inflammasome, which is a large multiprotein complex in the cytosol and regulates IL-1β production [4,6,7]. The inflammasome contains Nod-like receptors (NLRs) associated with an apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), which recruits caspase-1 and induces its activation. Since caspase-1 is an IL-1β -converting enzyme (ICE), it processes pro-IL-1β into mature IL-1β. These findings suggest the involvement of the inflammasome in the development of atherosclerosis. We have previously reported that ASC deficiency attenuates inflammatory responses and neointimal formation in the vascular walls in a murine model for vascular injury [8]. Furthermore, recent studies have shown that cholesterol crystals activate the inflammasome in macrophages, which may promote the development of atherosclerosis [7,9]. In contrast, Menu et al. [10] have reported that atherosclerosis progresses independent of the inflammasome. Although the reason for this discrepancy is unclear,

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the distinct role of the inflammasome in atherosclerosis remains controversial. In the present study, we evaluated the role of the inflammasome in atherosclerosis in apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice that developed spontaneous atherosclerotic lesions in the vascular walls due to hypercholesterolemia – a pattern similar to that observed in humans [11]. We produced the double deficient mice for *Apoe* and caspase-1 (*Apoe*$^{-/-}$*Casp1*$^{-/-}$). The male age-matched *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Casp1*$^{-/-}$ mice were fed a high-cholesterol diet (Western diet, WD) and were analyzed for atherosclerotic plaque formation and vascular inflammation. Since vascular calcification actively influences macrophages and promotes the plaque progression [12], we further investigated whether calcium phosphate crystals can activate caspase-1 and induce secretion of IL-1β and IL-1α in macrophages. The findings from this study demonstrate the critical role of caspase-1 in the development of atherosclerosis and provide a new insight into the role of the inflammasome in the pathogenesis of atherosclerosis.

2. Materials and methods

2.1. Animals and the development of atherosclerotic plaques

All experiments in this study were performed in accordance with the Jichi Medical University Guide for Laboratory Animals. C57BL/6J and *Apoe*$^{-/-}$ mice (male, 8-weeks-old; C57BL/6J background) were purchased from Clea Japan (Tokyo, Japan) and the Jackson Laboratory (Bar Harbor, ME), respectively. *Casp1*$^{-/-}$ mice (male, 8-weeks-old; C57BL/6 background) were kindly provided by Dr. Hiroko Tsutsui (Hyogo Medical College, Japan) [13,14]. *Apoe*$^{-/-}$*Casp1*$^{-/-}$ mice were produced by mating these mice. The mice were fed and watered and maintained on a 12-h light and dark cycle. Atherosclerotic plaques were developed when the mice were fed with WD (0.15% w/w cholesterol, 40 kcal% butter fat; D12079B, Research Diets, Inc., NJ) for 12 weeks.

2.2. Plasma lipid analyses

Plasma levels of total cholesterol (TC), triglycerides (TG), and nonesterified fatty acids (NEFA) after a 16-h fast were determined using colorimetric enzyme assay kits (Kyowa Medex Co. Ltd. for TC, and Wako Chemicals [Osaka, Japan] for TG and NEFA) according to the manufacturers' instructions.

2.3. Assessment of atherosclerosis in the whole aortas and aortic sinus

After the mice were euthanized, the aortas were excised and adventitial fat was removed. Aortas were then fixed in phosphate-buffered saline (PBS) containing 10% (w/v) formalin (Wako Chemicals). Whole aortas were opened longitudinally from the aortic arch to the iliac bifurcation, mounted *en face*, and stained for lipids with Sudan IV (Wako Chemicals) as previously described [15]. The extent of atherosclerotic areas was expressed as the percentage of lesion area in the entire aortic surface area. The hearts, perfused with PBS containing 10% (w/v) formalin, were embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical Co. Ltd., Tokyo, Japan), and 6-μm thick serial sections were cut using a cryostat (Leica CM1850; Leica Microsystems, Germany). The cross-sections of the aortic sinus were stained with Oil Red O (Sigma) and hematoxylin (Wako Chemicals). The average lesion area was measured using 10 sections from each mouse stained with Oil Red O. Images for atherosclerotic plaque areas were captured using the Leica Application Suite software (ver. 3.4.1, Leica Microsystems) and analyzed using the Adobe Photoshop CS4 software (Adobe Systems Inc., CA).

2.4. Immunohistochemistry

Immunohistochemical analysis was performed to detect IL-1β expression, macrophage infiltration, and VSMC composition in the plaques [15,16]. Briefly, the sections were incubated with primary antibody to IL-1β (R&D Systems), macrophage marker MOMA-2 (AbD Serotec, Raleigh, NC), and smooth muscle cell marker a-smooth muscle actin (α-SMA; clone 1A4, Sigma, St. Louis, MO). This was followed by incubation with biotin-conjugated secondary antibodies. The sections were washed and treated with avidin-peroxidase (ABC kit; Vector Laboratories, Burlingame, CA). The reaction was developed using a DAB substrate kit (Vector Laboratories). The sections were then counterstained with hematoxylin. No signals were detected when an irrelevant IgG (Vector Laboratories) was used instead of the primary antibody as a negative control. All measurements were conducted in a double-blind manner by 2 independent researchers.

2.5. Measurement of inflammatory cytokines

The levels of IL-1β, IL-1α, IL-6 CCL2 (MCP-1, monocyte chemoattractant protein-1), and tumor necrosis factor-a (TNF-a) were assessed using a mouse enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) and cytometric bead array (CBA) kit (BD, Biosciences). For the CBA analysis, flow cytometry (FACS Calibur, BD Biosciences) was used.

2.6. Cell cultures and in vitro experiments

To generate murine primary bone marrow-derived macrophages (BMDMs), bone marrow cells were isolated from the femurs and tibias of mice and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Dainippon Pharmaceutical Co., Osaka, Japan) and 15% conditioned medium of L929 cells (ATCC, Rockville, MD) for 7 days. Murine J774 macrophage cells were obtained from RIKEN Gene Bank (Tsukuba, Japan) and cultured in 10%FCS/DMEM. After serum starvations and priming with a low-dose lipopolysaccharide (LPS, 10 ng/mL) for 16 h, the cells treated with monosodium urate monohydrate (MSU; InvivoGen, San Diego, CA) and tricalcium phosphate (TCP, Sigma) crystals in the presence or absence of bafilomycin (Sigma), CA-074 Me (Wako Chemicals), and Z-Tyr-Val-Ala-Asp-fluoromethylketone (YVAD-FMK) (MBL, Nagoya, Japan) for 6 h. All other reagents were obtained from Sigma unless otherwise specified.

2.7. Measurement of caspase-1 activity

Caspase-1 activity was analyzed using the carboxyfluoroescein FLICA Caspase-1 assay kit (Immunochemistry Technologies, Bloomington, MN) according to the manufacturer's instructions. Nuclei were co-stained with Hoechst33342. Fluorescence was detected by using confocal laser scanning microscopy (FV-10i, Olympus, Tokyo, Japan).

2.8. Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM). An unpaired *t* test was used to compare 2 groups. For comparisons between multiple groups, the significance of differences between-group means was determined by one-way analysis of variance (ANOVA) combined with the Turkey–Kramer test. All analyses were performed using the GraphPad Prism Software (ver. 4, San Diego, CA). A p-value of <0.05 was considered statistically significant.