Inflammasome Activation of Cardiac Fibroblasts Is Essential for Myocardial Ischemia/Reperfusion Injury

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Background—Inflammation plays a key role in the pathophysiology of myocardial ischemia/reperfusion (I/R) injury; however, the mechanism by which myocardial I/R induces inflammation remains unclear. Recent evidence indicates that a sterile inflammatory response triggered by tissue damage is mediated through a multiple-protein complex called the inflammasome. Therefore, we hypothesized that the inflammasome is an initial sensor for danger signal(s) in myocardial I/R injury.

Methods and Results—We demonstrate that inflammasome activation in cardiac fibroblasts, but not in cardiomyocytes, is crucially involved in the initial inflammatory response after myocardial I/R injury. We found that inflammasomes are formed by I/R and that its subsequent activation of inflammasomes leads to interleukin-1β production, resulting in inflammatory responses such as inflammatory cell infiltration and cytokine expression in the heart. In mice deficient for apoptosis-associated speck-like adaptor protein and caspase-1, these inflammatory responses and subsequent injuries, including infarct development and myocardial fibrosis and dysfunction, were markedly diminished. Bone marrow transplantation experiments with apoptosis-associated speck-like adaptor protein–deficient mice revealed that inflammasome activation in bone marrow cells and myocardial resident cells such as cardiomyocytes or cardiac fibroblasts plays an important role in myocardial I/R injury. In vitro experiments revealed that hypoxia/reoxygenation stimulated inflammasome activation in cardiac fibroblasts, but not in cardiomyocytes, and that hypoxia/reoxygenation–induced activation was mediated through reactive oxygen species production and potassium efflux.

Conclusions—Our results demonstrate the molecular basis for the initial inflammatory response after I/R and suggest that the inflammasome is a potential novel therapeutic target for preventing myocardial I/R injury. (Circulation. 2011;123:594-604.)

Key Words: cytokine ■ heart ■ hypoxia ■ inflammation ■ leukocyte
some plays an important role not only in bone marrow–derived cells but also in resident cells of the myocardium in the development of I/R injury. Furthermore, we aimed to identify the mechanisms underlying inflammasome activation during myocardial I/R. Our study findings provide new insight into the role of inflammasome in the pathophysiology of myocardial I/R injury and suggest that the inflammasome is a potential novel therapeutic target for the prevention of myocardial I/R injury.

We next assessed LV function after myocardial I/R using echocardiography. WT and ASC−/− mice had similar LV dimension and function under baseline conditions (Figure 1D). In the WT mice, a marked decrease in percent fractional shorting was observed at 7 and 14 days after myocardial I/R. In contrast, percent fractional shortening was maintained after myocardial I/R in ASC−/− mice (Figure 1D). Furthermore, in ASC−/− mice, the diastolic and systolic LV dimensions were significantly lower at 7 and 14 days after myocardial I/R compared with baseline. Because the echocardiographic findings of ASC−/− mice indicated the beneficial effect of ASC deficiency on LV remodeling after myocardial I/R, Masson trichrome staining was performed to evaluate interstitial fibrotic changes in the myocardium. The fibrotic area/LV area percentage was significantly reduced in all of the scar, border, and perivascular areas of ASC−/− mice compared with WT mice (Figure 1E and 1F). As expected, the ratio of heart weight to body weight was also significantly decreased in ASC−/− mice compared with WT mice (Figure 1G).

ASC Expression, Inflammatory Cell Infiltration, Capillary Formation, and Apoptosis

Because ASC was markedly expressed in human MI (Figure 1A), we determined ASC expression in myocardial I/R in mice using immunohistochemical analysis. Consistent with ASC expression in human MI, ASC expression was clearly observed in the infiltrated mononuclear cells and neutrophils in the ischemic myocardium (Figure 2A). In addition, ASC was weakly expressed in fibroblast-like interstitial cells. In situ hybridization also revealed the expression of ASC messenger RNA in the ischemic myocardium (Figure 2B). To identify the cells expressing ASC in the ischemic myocardium, we performed double-immunofluorescence staining using antibodies against ASC, macrophages (Mac-3), and neutrophils (Ly6G) (Figure 2C). We found that almost all macrophages expressed ASC, whereas ≈60% of neutrophils expressed ASC (Figure 2D and Figure II in the online-only Data Supplement).

We then compared the infiltration of macrophages and neutrophils in WT and ASC−/− mice. The numbers of infiltrated both macrophages and neutrophils were significantly decreased in the ischemic and border areas of the myocardium in ASC−/− mice compared with WT mice (Figure 3A through 3D). We further assessed capillary density using immunohistochemical analysis of endothelial marker CD31. There was no significant difference in capillary density between WT and ASC−/− mice (Figure 3E and 3F). Supporting this finding, flow cytometry analysis showed no difference between WT and ASC−/− mice in the proportion of peripheral CD34+/Flk-1+ cells (an ordinary endothelial progenitor cell marker)15 after I/R injury (Figure 3G). In addition, although it has been suggested that inflammasomes are implicated in the process of apoptosis,16 there was no difference in the number of apoptotic cells, as determined by cleaved caspase-3 between WT and ASC−/− mice (Figure 3H).

Expression of Inflammatory Cytokines and Involvement of Apoptosis

To investigate whether inflammatory cytokines are involved in the reduction of myocardial I/R injury in ASC−/− mice, we assessed protein and messenger RNA levels of various inflam-
matory cytokines in the ischemic myocardium. The protein levels of IL-1β, tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), and IL-6, but not interferon-γ (IFN-γ) or IL-10, were higher in the ischemic myocardium of WT mice compared with control mice (sham operated) (Figure 4A). In the ischemic myocardium of ASC−/− mice, the levels of all these inflammatory cytokines were decreased compared with WT mice. Of note, near-peak expression of IL-1β and IL-18 was detected as early as 3 hours after I/R (Figure 4B). TNF-α was also increased at 3 hours after I/R, but there was no significant difference between WT and ASC−/− mice. Real-time reverse-transcriptase polymerase chain reaction analysis also showed elevated messenger RNA levels of IL-1β, IL-6, and MCP-1 in the ischemic myocardium of WT mice, whereas these elevations except for IL-18 and TNF-α were decreased in ASC−/− mice (Figure 4C).

**Contribution of Bone Marrow–Derived Cells**

Because ASC was highly expressed in infiltrated inflammatory cells derived from bone marrow, we explored the role of bone marrow–derived cells in vitro and in vivo. In vitro, we activated inflammasomes with a well-characterized stimulus,
lipopolysaccharide (LPS), and measured the production of various inflammatory cytokines in the supernatant of bone marrow cells. LPS treatment markedly increased the production of IL-1β, IL-6, MCP-1, IFN-γ, and TNF-α, but not IL-10, in bone marrow cells isolated from WT mice (Figure 5A). However, LPS-induced production of IL-1β, IL-6, MCP-1, and IFN-γ was significantly inhibited in bone marrow cells isolated from ASC/ASC/ASC mice. No significant difference was observed in LPS-induced TNF-α production between WT and ASC/ASC/ASC mice.

To determine the contribution of bone marrow-derived cells in vivo, we produced 3 types of BMT mice (BMTWT3WT, BMTASC/ASC/ASC3WT, and BMTWT3ASC/ASC/ASC mice). To verify the reconstitution of bone marrow after transplantation, we examined ASC expression in the splenocytes of the BMT mice. As expected, ASC expression was detected in both BMTWT→WT and BMTWT→ASC/ASC/ASC mice but not BMTASC/ASC/ASC→WT mice (Figure 5B). Despite the lack of difference in AAR/LV percentage, the infarct area/AAR percentage was significantly reduced in BMTASC/ASC/ASC→WT mice compared with BMTWT→WT (Figure 5C and 5D). An important point to note is that the infarct area/AAR percentage was also significantly reduced in BMTWT→ASC/ASC/ASC mice. This finding indicates the importance of ASC not only in bone marrow-derived cells but also in resident cells of the myocardium in I/R injury development.

Role of Caspase-1 in Myocardial I/R Injury
Caspase-1 participates in assembling the inflammasome complex with ASC and in the processing of pro-IL-1β to its mature form. To further verify the role of inflammasomes in myocardial I/R injury, we used caspase-1/−/− mice. Similar to the results obtained from ASC/−/− mice, the expression of caspase-1 was visualized mainly in the infiltrated cells of the ischemic myocardium (Figure 6A through 6C). Furthermore, the infarct area/AAR percentage was significantly reduced in caspase-1/−/− mice, similar to that observed in ASC/−/− mice (Figure 6D and 6E), indicating the critical role of inflammasomes in the development of myocardial I/R injury.

Inflammasome Activation in Cardiac Fibroblasts but Not in Cardiomyocytes
Consistent with prior observations, a histological time course representing the state of the myocardium after I/R revealed that myocardial damage and hemorrhage were observed as early as 3 hours after I/R and that subsequent infiltration of inflammatory cells occurred from 6 to 48 hours after I/R (Figure III in the online-only Data Supplement). Therefore, we hypothesized that cardiomyocytes or cardiac fibroblasts might be responsible for initial inflammasome activation in myocardial I/R. To test this hypothesis, we used murine neonatal-cultured cardiomyocytes and cardiac fibroblasts derived from WT and ASC/−/− mice in vitro. We confirmed that
ASC was surely expressed in cardiomyocytes and cardiac fibroblasts (Figure 7A). Although treatment with a high dose of LPS (100 ng/mL) did not alter the expression levels of ASC in either cardiomyocytes or cardiac fibroblasts (Figure 7B), LPS treatment clearly induced IL-1β production in cardiac fibroblasts although not in cardiomyocytes (Figure 7C through 7E). In addition, LPS-induced IL-1β production in ASC−/− cardiac fibroblasts was significantly lower than in WT cardiac fibroblasts (Figure 7D). Western blot analysis showed that stimulation with LPS directly activated inflammasomes, as indicated by the appearance of mature IL-1β (p17) in cardiac fibroblasts (Figure 7E). Quantitative analysis showed that LPS-induced IL-1β processing (a ratio of mature IL-1β to pro-IL-1β) was significantly decreased in ASC−/− cardiac fibroblasts compared with WT cardiac fibroblasts (Figure IV in the online-only Data Supplement).

Mechanism of Inflammasome Activation After I/R in the Myocardium
Most inflammasome activators trigger cellular intermediary signals. Common mechanisms implicated in inflammasome activation include ATP, a low concentration of intracellular...
Figure 4. Inflammatory cytokine expression. 
A, Heart samples were obtained from control (sham), ischemic area, and nonischemic area of WT and ASC−/− mice at 6 hours after myocardial I/R injury. Myocardial levels of IL-1β, IL-6, MCP-1, IFN-γ, TNF-α, and IL-10 were assessed. Data are expressed as mean±SEM (n=5 for each). *P<0.05 and **P<0.01 vs WT. B, Heart samples were obtained from control (sham), ischemic area, and nonischemic area of WT and ASC−/− mice at 3 hours, 6 hours, and 14 days after myocardial I/R injury. Myocardial levels of IL-1β, IL-18, and TNF-α were assessed. Data are expressed as mean±SEM (n=3 for each). C, Heart samples were obtained from control (sham), ischemic area, and nonischemic area of WT and ASC−/− mice after myocardial I/R injury at the indicated times. Myocardial messenger RNA levels of IL-1β, MCP-1, IL-6, IL-18, and TNF-α were assessed by reverse-transcriptase polymerase chain reaction analysis. Data are expressed as mean±SEM (n=4 for each). Data were analyzed by 2-way ANOVA followed by t test. *P<0.05 and **P<0.01 vs WT.
potassium,19,20 and production of reactive oxygen species (ROS).9,21 To test whether I/R can activate inflammasomes in cardiomyocytes or cardiac fibroblasts, we stimulated the cells with H/R. Because pro-IL-1β is not constitutively expressed and requires transcriptional induction, we primed the cells with a low dose of LPS (10 ng/mL), as in published studies,7,9 to ensure induction of pro-IL-1β. H/R clearly induced a robust production of IL-1β and ROS production in cardiac fibroblasts but not in cardiomyocytes (Figure 8A and Figure V in the online-only Data Supplement). These findings suggest that inflammasome activation in cardiac fibroblasts is essential for the inflammatory response to heart I/R.

To investigate the factor(s) responsible for activation of the inflammasome in cardiac fibroblasts, various stimuli, including ATP, nigericin (K+ ionophore), hydrogen peroxide (H2O2), high-mobility group box 1 (HMGB1; a danger signal secreted by the ischemic myocardium), and cobalt chloride (CoCl2; a chemical hypoxia inducer), were added to the culture medium of cardiac fibroblasts; the production of IL-1β was then measured. A significant production of IL-1β was detected after nigericin and H/R stimulation, but not by ATP, HMGB1, CoCl2, and H2O2 stimulation (Figure 8B). Furthermore, the production of IL-1β, but not TNF-α, stimulated by nigericin or H/R was almost completely extinguished in ASC−/− cardiac fibroblasts (Figure 8C and Figure VI in the online-only Data Supplement). H/R also stimulated IL-18 production, and this production was inhibited in ASC−/− cardiac fibroblasts (Figure VII in the online-only Data Supplement). To block K+ efflux in cardiac fibroblasts, we treated the cells with high concentrations (130 mmol/L) of KCl or potassium channel blockers such as glimepiride, glibenclamide, and tetraethylammonium and demonstrated that inhibition of K+ efflux blocked

Figure 5. Contribution of bone marrow–derived cells. A, Bone marrow cells were isolated from WT and ASC−/− mice and incubated for 24 hours in the presence or absence of LPS (100 ng/mL). Levels of IL-1β, IL-6, MCP-1, IFN-γ, TNF-α, and IL-10 in the supernatants were assessed. Data are expressed as mean±SEM (n=8 for each). Data were analyzed by 2-way ANOVA followed by t test. *P<0.05, **P<0.01, ***P<0.001. B, BMTWT→WT, BMTWT→ASC−/−, and BMTASC−/−→WT mice were developed, and myocardial I/R was produced in them 8 weeks after BMT. Splenocytes were isolated and analyzed by Western blotting with antibodies against ASC and β-actin (n=2). C, Heart sections were obtained from BMT mice (BMTWT→WT, BMTWT→ASC−/−, and BMTASC−/−→WT) at 48 hours after myocardial I/R injury. D, Quantitative analysis of AAR/LV area percentage and infarct area/AAR area percentage was performed. Representative photographs are shown. Data are expressed as mean±SEM (n=8 for each). Data were analyzed by repeated measures ANOVA.
H/R-dependent IL-1β production (Figure 8D). Furthermore, N-acetyl-cysteine (an antioxidant) decreased IL-1β production. Measurement of K⁺ concentration in the culture medium further supported the role of K⁺ efflux in inflammasomes after H/R (Figure VIII in the online-only Data Supplement). In addition, the expression of potassium channel subunits was confirmed in cardiac fibroblasts (Figure IX in the online-only Data Supplement). Taken together, these findings suggest that I/R activation of inflammasomes might be mediated through ROS production and K⁺ efflux.

Discussion

The major findings of this study are as follows: (1) ASC and caspase-1 were clearly expressed at the site of myocardial I/R injury; (2) ASC and caspase-1 deficiency reduced inflammatory responses such as inflammatory cell infiltration and cytokine expression and subsequent injuries such as infarct development, myocardial fibrosis, and dysfunction in myocardial I/R injury; (3) BMT experiments conducted with ASC-deficient mice showed that inflammasome activation in bone marrow cells and myocardial resident cells plays an important role in myocardial I/R injury; and (4) H/R stimulated inflammasome activation in cardiac fibroblasts, but not in cardiomyocytes in vitro, and H/R-induced activation was mediated through ROS production and potassium efflux. The results of the present study clearly indicate that inflammasome activation in cardiac fibroblasts plays an important role in inflammatory responses and subsequent injury after myocardial I/R, and the activation is partly mediated by potassium efflux and ROS production. These findings clarify the molecular events occurring during the initial inflammatory response after myocardial I/R and suggest that the inflammasome is a potential novel therapeutic target for myocardial I/R injury.

Increasing evidence indicates the importance of inflammation in the pathophysiology of myocardial I/R injury. For instance, interventions targeted at leukocytes or inflammatory mediators substantially reduce myocardial I/R injury. In particular, the neutralization of IL-1β reduces injury, suggesting that IL-1β is the key mediator in the pathophysiology of myocardial I/R injury. This finding is supported by the observed reduction in injury after MI in the absence of caspase-1 activity. However, recent evidence indicates that sterile inflammation is triggered by tissue damage, and
endogenous danger signals are mediated by the inflammasome.8–12 Thus, we hypothesized that the inflammasome is an initial sensor in myocardial I/R injury and showed that ASC and/or caspase-1 deficiency considerably improves inflammatory responses and subsequent injury after myocardial I/R. These findings highlight the importance of the inflammasome in the initial inflammatory response to myocardial I/R injury.

Because ASC is highly expressed in the infiltrated cells of the ischemic myocardium, it is expected that ASC plays a substantial role in bone marrow–derived cells. Indeed, in vitro experiments revealed that the production of inflammatory cytokines is decreased in ASC-deficient bone marrow cells. Furthermore, BMT experiments revealed the importance of inflammasome activation in bone marrow–derived cells in myocardial I/R injury. In the present study, ASC deficiency in non–bone marrow–derived cells unexpectedly resulted in a reduction in infarct area after myocardial I/R injury; this reduction was similar to that induced by ASC deficiency in bone marrow–derived cells. This observation suggests that myocardial resident cells such as cardiomyocytes or cardiac fibroblasts contribute to the initial activation of the inflammasome after I/R. This hypothesis is supported by findings of the present and other studies. First, we observed myocardial damage at an early stage before the infiltration of inflammatory cells. Second, IL-1β expression almost peaked at the same early time point. Third, myocardial I/R injury has been experimentally induced in isolated hearts perfused with crystalloid solutions that lack bone marrow–derived cellular components.24 In the present study, we observed that IL-1β was detected only in cardiac fibroblasts, but not in cardiomyocytes, although ASC was expressed in both cardiomyocytes and cardiac fibroblasts. In addition, in vitro experiments conducted with ASC-deficient cardiac fibroblasts revealed that inflammasome activation is essential for an initial inflammatory response in myocardial I/R injury. Cardiac fibroblasts make up as much as two thirds of the cell population in cardiac tissue and are a major component of the heart.25 Unlike cardiomyocytes, cardiac fibroblasts can proliferate even in the adult heart and produce extracellular matrix proteins (eg, collagen and fibronectin), cytokines, and growth factors and affect cardiac repair, hypertrophy, and remodeling. The present study highlights a new role of cardiac fibroblasts as “sentinel” cells that sense danger signals and enhance the inflammatory response in I/R.

The molecular basis of inflammasome activation in myocardial I/R is completely uncharacterized. Because ROS production and potassium efflux have been linked to the activation of inflammasomes in response to diverse inflammatory danger signals, including ATP, urate crystals, and asbestos or silica,7–9
we propose that I/R induces ROS production and potassium efflux in cardiac fibroblasts. These effects in turn activate inflammasomes and induce subsequent IL-1β production, thereby resulting in the initial inflammatory response in the myocardium. This initial inflammatory response stimulates the release of chemokines, which recruit inflammatory cells such as monocytes/macrophages and neutrophils to the ischemic myocardium. Furthermore, inflammasome activation in the infiltrated inflammatory cells enhances myocardial I/R injury.

Several limitations of this study should be noted. First, although the present study demonstrated that inhibition of initial inflammation improved cardiac function and remodeling after I/R, inflammation in myocardial I/R has been shown to be not only deleterious but also beneficial in the process of myocardial damage and remodeling. In particular, a key inflammatory mediator, TNF-α, is reported to have a paradoxical effect such as promoting or protecting in myocardial I/R injury. Second, although the inflammatory response after MI in mice shares many common characteristics with higher mammalian species and is an excellent model for exploration of cellular and molecular mechanisms, we should understand the potential limitations of extrapolating data from mice to humans. Third, although primary culture of neonatal cardiac cells has been widely used to study the mechanism of cardiovascular disorders, differences may exist between neonatal and adult cardiac cells. Thus, further investigations are necessary to understand the precise role of inflammasome in myocardial I/R injury.

Conclusions
This study is the first to demonstrate the essential role of the inflammasome in the pathophysiology of myocardial I/R injury. Our findings have also revealed that cardiac fibroblasts may mediate inflammation in response to I/R. Moreover, our results suggest that the inflammasome is a novel therapeutic target for preventing myocardial I/R injury.

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Disclosures
None.

References
Accumulating reports have demonstrated the important role of an inflammation-induced multiple-protein complex called the inflammasome in the field of immunology. To date, however, little is known about the role of inflammasome in the pathophysiology of myocardial ischemia/reperfusion (I/R) injury. Activation of inflammasome is triggered by tissue damage and leads to the processing of prointerleukin-1β (interleukin-1β) to its mature form. In the present study, we demonstrate that inflammasome activation in cardiac fibroblasts, but not in cardiomyocytes, is crucially involved in the initial inflammatory response after myocardial I/R injury. We found that inflammasomes are formed by I/R and that its subsequent activation of inflammasomes leads to interleukin-1β production, resulting in inflammatory responses such as inflammatory cell infiltration and cytokine expression in the heart. In mice deficient for apoptosis-associated speck-like adaptor protein (caspase-1), these inflammatory responses and subsequent injuries, including infarct development and potassium efflux. Our results demonstrate the molecular basis for the initial inflammatory response after I/R and suggest that the inflammasome is a potential novel therapeutic target for preventing myocardial I/R injury.
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Materials and Methods

Experimental animals and human samples

All animal experiments in this study were performed in accordance with the Shinshu University Guide for Laboratory Animals that conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publications No. 85-23, revised 1996). ASC-deficient (ASC−/−) mice were generated and used as described previously.1,2 They were then backcrossed to the C57BL/6 strain for at least ten generations. These mice were maintained in our animal care facility and intercrossed with the C57BL/6 strain mice. The resulting littermates were used for this study (wild-type [WT]: ASC+/+ littermates and homozygous ASC−/− mice, male, 12–14 weeks old). Other WT mice (C57BL/6, male, 12–14 weeks old) were purchased from Japan SLC Inc. (Hamamatsu, Japan). Caspase-1−/− mice were kindly provided by Hiroko Tsutsui (Hyogo Medical College, Japan).3,4 All animals were fed a standard diet and water and were maintained on a 12-h light and dark cycle. To determine the expression of ASC in human MI heart, the autopsy heart samples were obtained from a 59-year-old acute MI patient.

Murine model of ischemia/reperfusion (I/R) injury

WT and ASC−/− mice were anesthetized and subjected to myocardial I/R. Briefly, after the mice were ventilated with a rodent ventilator (MiniVent Type 845, Harvard Apparatus, Holliston, MA), a left thoracotomy was performed. After a 30-min ligation of the left anterior descending (LAD) coronary artery, reperfusion was established for the indicated period. The area at risk (AAR) and infarct area were determined by perfusion with 1% Evans blue and
consequent incubation with 1.5% triphenyltetrazolium chloride (TTC) at 48 h after the I/R. Each heart was digitally recorded with a microscope and a digital camera (DSL-F717, Cyber Shot, Sony, Tokyo, Japan). The left ventricle (LV) area, AAR, and infarct area were determined with computer-assisted planimetry software (Image J; National Institute of Health, Bethesda, MD).

Echocardiography

Transthoracic echocardiography was performed using a Vivid Five system (GE Yokogawa Medical Systems, Tokyo, Japan) as described previously. In brief, ketamine (50 mg/kg) and xylazine (10 mg/kg) were administered intraperitoneally for mild sedation. Two-dimensional targeted M-mode echocardiograms were obtained along the short axis of the LV at the level of the papillary muscles, and at least three consecutive beats were evaluated. The phases in which the smallest and largest area of the LV were obtained were defined as the LV end-systolic diameter (LVDs) and LV end-diastolic diameter (LVDd), respectively. The percentage fractional shortening (%FS) was calculated using the standard formula: \( \%\text{FS} = \left( \frac{\text{LVDd} - \text{LVDs}}{\text{LVDd}} \right) \times 100 \). All measurements were performed in a double-blind manner by two independent researchers.

Histology and immunohistochemistry

To perform histological and immunohistochemical analyses, the hearts were embedded in the OCT compound (Tissue-Tek; Miles Laboratories, Elkhart, IN) and frozen in liquid nitrogen, or fixed in zinc formalin and embedded in paraffin. Cryosections were cut 10 \( \mu \text{m} \) thick on a cryostat (CM-1900; Leica Microsystems GmbH, Wetzlar, Germany) and paraffin-embedded sections were cut 4 \( \mu \text{m} \) thick and histologically examined. The sections were stained with hematoxylin-eosin (HE) and Masson’s trichrome (MT). The extent of fibrosis in the sections was measured, and the value was expressed as the ratio of the MT-stained area to total LV area. Each image was digitized and analyzed under a microscope (BX-51; Olympus, Tokyo, Japan) using Image J software (National Institute of Health). All the measurements were
performed in a double-blind manner by two independent researchers.

For immunohistochemical analysis, the paraffin-embedded heart sections were incubated with primary antibodies against human and mouse ASC\textsuperscript{1,6,7}, human CD15 (clone MMA; BD Bioscience, San Jose, CA), human CD68 (clone KP-1; DAKO, Glostrup, Denmark), human CD3 (clone LN10; Leica Microsystems, Germany), human CD20 (clone L26; DAKO), mouse Ly6C (clone RB6-8c5; eBioscience, San Diego, CA), Mac-3 (clone M3/84; BD Bioscience), mouse F4/80 (clone A3-1; RDI, Flanders, NJ), mouse CD31 (clone MEC13.3; BD Bioscience), mouse interleukin (IL)-1\(\beta\) (R&D Systems, Minneapolis, MN), mouse caspase-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and cleaved caspase-3 (Asp-175; Cell Signaling Technology, Danvers, MA), followed by incubation with biotin-conjugated secondary antibodies and then treated with avidin-peroxidase (ABC Kit; Vector Laboratories, Burlingame, CA). The reaction was developed using the DAB substrate kit (Vector Laboratories). The sections were then counterstained with hematoxylin. No signals were detected when species-matched IgG (Vector Laboratories) was used instead of the primary antibody as a negative control. For immunofluorescence staining, Cy3-labeled goat anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG were used. The sections were also stained with 4',6-diamidino-2-phenylindole (DAPI; Wako Pure Chemical Industries Ltd., Osaka, Japan) for visualization of the nuclei. Immunofluorescence was observed by confocal laser scanning microscopy (Leica TCS-SP2 AOBS spectral laser scanning confocal microscopy system; Leica Microsystems, Heidelberg, Germany). Quantitative assessment of neutrophil, macrophage and capillary density was performed by counting the number of positive cells in five different fields (mm\textsuperscript{2}) of the area per heart. All measurements were performed in a double-blind manner by two independent researchers.

\textit{In situ hybridization (ISH)}

A digoxigenin (DIG)-labeled RNA probe was used to detect mouse ASC mRNA for
ISH.\textsuperscript{2} Aliquots of 1mg total RNA derived from C57BL/6 mouse spleen were reverse-transcribed and amplified by PCR using SuperScriptIII (Invitrogen, Carlsbad, CA) with mASC-specific reverse (5'-GTGCTCGAGGCTCTGCTCCAGGTCCATCAC-3') and forward (5'-CTGGGTACCTACCAGGCAGTTCGTGCAGAG-3') primers, where \textit{XhoI} and \textit{KpnI} sites are indicated with underline. The amplicon was then inserted into the \textit{XhoI} and \textit{KpnI} sites of pSPT18 vector (Roche Diagnostics, Mannheim, Germany), and the correct sequence was confirmed using an ABI3100 DNA sequencer (Applied Biosystems). A DIG-labeled antisense RNA probe was obtained from \textit{HindIII}-cut template and T7 RNA polymerase with a DIG RNA Labeling Mixture (Roche). Similarly, a control sense probe was generated using \textit{EcoRI}-cut template and SP6 RNA polymerase. Linearized probes were precipitated with LiCl-ethanol, dissolved in DEPC-water, and stored at \textdegree{}80°C until use.

Hybridization and detection of the probes were performed. Briefly, 7 mm-thick mouse tissue sections were deparaffinized in Hemo De (Falma Co., Japan), hydrated with ethanol, neutralized with 0.2 M HCl for 20 min, treated with 50 mg/mL proteinase K (Amresco, OH) at 37°C for 30 min, and then postfixed with 4% paraformaldehyde. Tissue slides were rinsed in 0.2% glycine and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0). Hydrated slides were defatted with chloroform and air-dried. Sections were prehybridized with 50% deionized formamide/2x SSC at 45°C for 1 h and then hybridized with 10 mg/mL of ASC antisense or sense probes in 50% deionized formamide, 2.5 mM EDTA (pH8.0), 0.3 M NaCl, 1x Denhardt’s solution, 10% dextran sulfate, and 1 mg/mL brewer’s yeast tRNA at 45°C for 48 h.

After hybridization, the tissue slides were washed in 50% formamide/2x SSC for 1 h at 45°C and digested with 10 mg/mL RNase A (Amresco) at 37°C for 30 min, followed by washing in 50% formamide/2x SSC and then by 50% formamide/1x SSC at 45°C for 1 h each. After washing, the tissue sections were subjected to immunohistochemistry to detect hybridized probes with an alkaline phosphatase-conjugated anti-DIG antibody (Roche). The alkaline phosphatase reaction was performed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium in the presence of 10% polyvinyl alcohol at 4°C overnight.
**Flow cytometry analysis**

Blood samples were collected from the mice and analyzed using flow cytometry. Circulating cells were identified using the nucleated cell fraction. The nucleated cells were double-labeled with the following antibodies: FITC-conjugated anti-CD34 (clone RAM34; BD Biosciences) and phycoerythrin (PE)-conjugated anti-Flk-1 (BD Biosciences). The cells were examined by flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ) and analyzed using CellQUEST software version 3.3 (Becton Dickinson).

**Measurement of inflammatory cytokines**

The levels of monocyte chemoattractant protein-1 (MCP-1), IL-6, IL-10, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) were assessed using the CBA Mouse Inflammation Kit (BD Biosciences) according to the manufacturer’s instructions. The level of IL-1β and IL-18 was assessed using a mouse ELISA kit (R&D Systems) according to the manufacturer’s instructions.

**Real-time RT-PCR analysis**

Total cellular RNA was extracted separately from the ischemic and the remote non-ischemic myocardium of WT and ASC−/− mice using ISOGEN (Nippon Gene Co. Ltd., Toyama, Japan), according to the manufacturer’s instructions. Real-time RT-PCR analysis was performed by using the Takara Real-time PCR system (Takara Bio, Shiga, Japan) to detect the mRNA expression. The following primers (oligonucleotide sequences are provided in parentheses in the order of antisense and sense primers) were used: IL-1β (5′-TGAAGTTGACGGACCCCAAA-3′ and 5′-TGATGTGCTGCTGTGAGATT-3′), IL-6 (5′-ACACCACGGCCTCCCTACTT-3′ and 5′-CACGATTTCCCAGAGACCATGTG-3′), MCP-1 (5′-GGCTCAGCCAGATGCAGTTAAC-3′ and 5′-GCCTACTCATTGGGATCATCTTG-3′).
IL-18 (5\′-CAGGCCTGACATCTTCGCAA-3\′ and 5\′-TCTGACATGGCAGCCATTGT-3\′), TNF-α (5\′-AAGCCTGTAGCCACGTCGTA-3\′ and 5\′-GGCACCACTAGTTGTTGCTTTG-3\′), Kir5\′ (5\′-CACAAGAACATCCGAGCA-3\′ and 5\′-CGTGAATGACCTGACATTGG-3\′), SUR2 (5\′-GAAGCCTTGGCTCCTAAATG-3\′ and 5\′-GAATGGGTCGTCCAAAAGA-3\′), and β-actin (5\′-CCTGAGCGCAAGTACTCTGTGT-3\′ and 5\′-GCTGATCCACATCTGCTGGAA-3\′). The expression levels of each target gene were normalized by subtracting the corresponding β-actin threshold cycle (C_T) values by using the ΔΔCT comparative method.

**Bone marrow transplantation (BMT)**

Whole bone marrow cells were harvested by flushing femurs with phosphate-buffered saline (PBS). Red blood cells were lysed with ACK buffer (150 mM NH_4Cl, 10 mM KHCO_3, 0.1 mM EDTA, pH 7.2) at 4˚C for 20 min. The cells were washed three times with PBS and resuspended in 0.5 mL PBS. Recipient mice (age: 8 weeks old) were lethally irradiated with a total dose of 9 Gy (MBR-155R2; Hitachi, Japan) and were injected with bone marrow cells through the tail vein. To verify the reconstitution of bone marrow after transplantation by this protocol, we used green fluorescent protein (GFP) mice as the donors. Flow cytometry analysis showed that at 6 weeks after BMT, peripheral blood cells consisted of more than 90% GFP\(^+\) cells. Using this protocol, we produced three types of BMT mice: WT to WT (BMT\(^{WT\rightarrow WT}\)) mice, WT to ASC\(^−/−\) (BMT\(^{WT\rightarrow AS C\rightarrow−}\)) mice, and ASC\(^−/−\) to WT (BMT\(^{ASC\rightarrow−\rightarrow WT}\)) mice.

**Cell culture and hypoxia/reoxygenation (H/R)**

Murine neonatal cardiomyocytes and cardiac fibroblasts were prepared from the ventricles of 1-day-old mice with minor modifications of the protocol used for rat neonatal cardiomyocyte isolation. Briefly, after dissociation with 0.25% trypsin (Invitrogen, Carlsbad, CA) followed by 0.8 mg/mL collagenase (Wako Pure Chemical Industries, Inc., Osaka, Japan),
the cells were washed and resuspended in Dulbecco’s modified Eagle’s medium (DMEM: Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS: Hyclone, Logan, UT). For isolation of cardiac fibroblasts, the cells were plated onto culture dishes for 1 hour, during which time cardiac fibroblasts readily attached to the bottom of the culture dishes. The non-attached cells (cardiomyocytes) were removed and plated onto other culture dishes. The isolated cardiomyocytes and cardiac fibroblasts were grown in 10% FBS-containing DMEM. Primary cardiomyocytes and cardiac fibroblasts were used in the experiments. To expose the cells to hypoxia, they were placed in Aneropac (Mitsubishi Gas Chemical, Tokyo, Japan). To verify the hypoxic condition, we measured pO2 of the culture media under the hypoxic condition by using Radiometer ABL700 (Radiometer Co., Tokyo, Japan). The levels of pO2 under the normoxic and hypoxic conditions were 140.7 ± 1.2 mmHg and 67.5 ± 1.5 mmHg, respectively (n = 3 in each, p < 0.0001).

To perform a positive immunostaining against ASC, THP-1 cells were obtained from the American Type Culture Collection (Rockville, MD) and differentiated with macrophages with phorbol 12-myristate 13-acetate (PMA, Sigma).

**Western blot analysis**

Expression of ASC and IL-1b was analyzed by western blotting. Cells were washed with ice-cold PBS and then lysed in RIPA buffer (25 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 10 mM sodium pyrophosphate, 50 mM NaF, 137 mM NaCl, 100 µM Na3VO4, 1% Triton X-100, 10% glycerol, 1% deoxycholic acid, 0.1% SDS, and fresh 0.1 mM phenylmethyl sulfonyl fluoride (PMSF) and 10 mg/mL of leupeptin). The cell lysates were prepared by scraping, sonication, and centrifugation. Cellular protein concentrations were determined by the DC protein assay (Bio-Rad). Cell lysates were subjected to SDS-PAGE under reducing conditions, and the protein bands then transferred to a nitrocellulose membrane. The membrane was blocked for 2 hours at room temperature with 5% skim milk, and then incubated for 1 hour at room temperature with the primary antibodies, followed by incubation for 1 hour with the secondary antibody, conjugated horseradish peroxidase. The primary antibodies against ASC,
IL-1β (R&D Systems), β-actin (Sigma, St. Louis, MO), and TFIIF (Santa Cruz Biotechnology, Inc.) were used. Immunoreactive bands were visualized by an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK). The expression levels of β-actin or TFIIF served as an internal control for protein loading.

References


**Supplementary figure legends**

**Supplementary figure 1. Negative control staining for the MI sample**

Human heart sections were obtained from acute MI patients and negative control staining was performed. To obtain a positive and negative immunostaining against ASC, we used PMA-treated THP-1 cells.

**Supplementary figure 2. ASC expression in the myocardium**

Heart sections were obtained from the myocardium of the sham-operated WT mice. The sections were immunohistologically analyzed by staining with antibody against ASC. Representative photographs are shown (n = 3).

**Supplementary figure 3. Time course of inflammatory cell infiltration after I/R injury**

Heart sections were obtained from WT mice at the indicated times after myocardial I/R injury. The sections were stained with HE. Representative photographs are shown (n = 3).

**Supplementary figure 4. Pro-IL-1β processing induced by LPS in WT- and ASC−/−-cardiac fibroblasts**

WT- and ASC−/−-cardiac fibroblasts were treated with LPS (100 ng/mL) for 24 h and analyzed by western blotting with antibodies against IL-1β and β-actin. Quantitative analysis was performed to measure the relative expressions of pro-IL-1β/β-actin, mature IL-1β/β-actin, and mature IL-1β/pro-IL-1β. Data are expressed as mean ± SEM (n = 3).

**Supplementary figure 5. ROS production in cardiac fibroblasts**

Cardiac fibroblasts were pretreated with or without NAC (10 mM), and then stimulated with H/R. The levels of ROS production were determined with dihydroethidium (DHE) fluorescent staining. Representative photographs were shown (n = 3).
**Supplementary figure 6. TNF-α production in cardiomyocytes and cardiac fibroblasts**

WT- and ASC−/−-derived cardiomyocytes and cardiac fibroblasts were stimulated with H/R in the presence or absence of a low dose LPS (10 ng/mL). The levels of TNF-α in the supernatants were assessed. Data are expressed as mean ± SEM (n = 3).

**Supplementary figure 7. IL-18 production in cardiac fibroblasts**

WT- and ASC−/−-derived cardiac fibroblasts were stimulated with H/R in the presence or absence of a low dose LPS (10 ng/mL). The levels of IL-18 in the supernatants were assessed. Data are expressed as mean ± SEM (n = 3).

**Supplementary figure 8. Extracellular potassium concentration in the supernatant of cardiac fibroblasts**

LPS (10 ng/mL)-primed cardiac fibroblasts were treated with or without NAC (10 mM) or nigericin (1 µM) under normoxia or hypoxia for the indicated periods. Extracellular potassium concentrations in the supernatants were assessed. Data are expressed as mean ± SEM (n = 3).

**Supplementary figure 9. Expression of potassium channel subunits in cardiac fibroblasts**

Expression of potassium channel subunits (Kir6.1 and SUR2) in adult and neonatal cardiac fibroblasts was analyzed by RT-PCR analysis. GAPDH served as the internal control (n = 3).
Sham-operated heart

Control IgG

ASC

500 µm  20 µm
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Supplementary figure 3.
Supplementary figure 4.

WT ASC<sup>−/−</sup>

Pro IL-1β

Mature IL-1β

β-actin

Pro IL-1β/β-actin ratio

Mature IL-1β/β-actin ratio

P < 0.01

WT ASC<sup>−/−</sup>

Mature IL-1β/pro IL-1β ratio

P < 0.01
Supplementary figure 6.

The graph shows the effect of LPS (10) and H/R on TNF-α expression in CMs and CFs. The conditions tested are Nil, LPS (10), H/R, LPS (10) + H/R, Nil, LPS (10), H/R, and LPS (10) + H/R. The graph compares WT and ASC−/− cells. The x-axis represents the conditions, and the y-axis represents TNF-α (pg/mg protein) levels. Statistical significance is indicated by asterisks: * p < 0.05, ** p < 0.01.
Supplementary figure 7.

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Control
LPS
H/R
LPS+H/R

WT
ASC−/−

P < 0.01

IL-18 (pg/mg protein) vs. WT and ASC−/−