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Nrf2 in bone marrow-derived cells positively contributes to the advanced stage of atherosclerotic plaque formation

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ABSTRACT

Atherosclerosis is the major etiology underlying myocardial infarction and stroke, and strategies for preventing atherosclerosis are urgently needed. In the context of atherosclerosis, the deletion of the *Nrf2* gene, which encodes a master regulator of the oxidative stress response in mammals, reportedly attenuates atherosclerosis formation. However, the precise mechanisms of protection against atherosclerosis are largely unknown. To further clarify the role of Nrf2 in atherosclerosis in vivo, we performed a time course analysis of atherosclerosis development utilizing an *ApoE* knockout (KO) mouse model. The results demonstrate that oil red O-stainable lesions were similar in size 5 weeks after the initiation of an HFC (high fat and high cholesterol) diet, but the lesions were markedly attenuated in the *Nrf2* and *ApoE* double KO mice (A0N0 mice) compared with the lesions in the *ApoE* KO mice (A0N2 mice) at 12 weeks. Consistent with these results, the immunohistochemical analysis revealed that Nrf2 activation is observed in late-stage atherosclerotic plaques but not in earlier lesions. The RT-qPCR analysis of 12-week atherosclerotic plaques revealed that Nrf2 target genes, such as *Ho-1* and *SLPI*, are expressed at significantly lower levels in the A0N0 mice compared with the A0N2 mice, and this change was associated with a decreased expression of macrophage M1-subtype genes Arginase II and inducible NO synthase in the A0N0 mice. Furthermore, the bone marrow (BM) transplantation (BMT) analysis revealed that the Nrf2 activity in the BM-derived cells contributed to lesion formation. Therefore, our study has characterized the positive role of Nrf2 in the BM-derived cells during the development of atherosclerosis, which suggests that Nrf2 may influence the inflammatory reactions in the plaques.

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Introduction

Atherosclerosis is not only a lipid metabolism disorder, but it is also a chronic inflammatory disease of the vasculature. Atherosclerosis is the major etiology of myocardial infarction and stroke and is responsible for a large percentage of all deaths in developed countries. A high blood cholesterol level, especially high serum levels of LDL, and inflammatory reactions in the blood vessels are critical in the initiation of atherosclerosis. During the initiation of atherosclerosis, blood monocytes migrate into the intima via the interaction with inflammatory cell adhesion molecules, such as VCAM-1 and ICAM-1, which are expressed on the endothelial cells during endothelial inflammation. Migrated monocytes differentiate

Abbreviations: Nrf2, NF-E2-related factor 2; BM, bone marrow; BMT, bone marrow transplantation; LDL, low density lipoprotein; CM, chylomicron; HDL, high-density lipoprotein; TG, triglyceride; CHO, cholesterol; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular cell adhesion molecule 1; oxLDL, oxidized LDL; VLDL, very low density lipoprotein; SLPI, secretory leukocyte protease inhibitor; HFC diet, high fat and high cholesterol diet; Ho-1, heme oxygenase-1; ApoE, apolipoprotein E; iNOS, inducible nitric oxide synthase; ArgI, arginase I; ArgII, arginase II; IL-6, interleukin-6; TNF-α, tumor necrosis factor α; Txnrd1, thioredoxin reductase 1; Srxn1, sulfiredoxin 1 homolog; Nqo1, NAD(P)H dehydrogenase, quinone 1; Gclm, glutamate-cysteine ligase, modifier subunit; WT, wild type; KO, knockout; A0N2, ApoE KO and Nrf2 WT; A0N0, ApoE KO and Nrf2 KO

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into macrophages and transform into foam cells by uptaking oxidized lipids; these transformed cells manifest as fatty streaks in the blood vessels [1–4].

Atheroprotective laminar flow (i.e., high laminar flow or L-flow) possesses anti-inflammatory properties that repress the expression of proinflammatory genes, such as VCAM-1, in endothelial cells [5,6]. Our group and other researchers have previously shown that the transcription factor Nrf2 is activated specifically by the L-flow [7–10]. Athero-prone regions, such as bends and bifurcations of the aorta, lack this anti-inflammatory mechanism, which partially explains their susceptibility to atherosclerosis [6]. Subsequent studies also demonstrated that Nrf2 is specifically activated in the atheroprotective regions (the region exposed to high laminar flow) in vivo, and *Nrf2* KO mice show high levels of VCAM-1 in the originally atheroprotected regions [10].

It has become widely accepted that macrophages display heterogeneity in the atherosclerotic plaques during the progression of atherosclerosis [4,11,12]. Plaque-associated macrophages can undergo pro- (i.e., M1 type) and anti-inflammatory (i.e., M2 type) polarization depending on the environmental signals [11]. Recently, the so-called Mox macrophages, which are oxidized lipid-inducible macrophages, have also been found in atherosclerotic lesions [12]. However, the roles of these heterogenic macrophages in the development of atherosclerosis are not yet clear. In addition to the above-noted atheroprotective function of Nrf2 in the endothelial cells, we previously demonstrated that Nrf2 enhances oxLDL uptake and foam cell formation in macrophages by directly upregulating the scavenger receptor CD36 in vitro [13]. Furthermore, several recent studies demonstrated that Nrf2 promotes atherosclerosis formation in vivo [14–16]. However, the functions of Nrf2 in atherosclerosis development are largely unknown. To clarify the roles of Nrf2 in atherosclerosis in more detail, we utilized an *ApoE* KO mouse model combined with the BMT technique. As a result, we have clarified the role of Nrf2 in BM-derived cells in atherosclerosis.

Materials and methods

Mice

Nrf2 KO mice from a C57BL/6J background were produced as previously described [17]. *ApoE* KO mice were obtained from The Jackson Laboratory. To accelerate atherosclerosis, the mice were fed a high fat diet containing 1.25% cholesterol (HFC diet; Research Diet Inc., New Brunswick, NJ, USA) for 3, 5, or 12 weeks beginning at 4 weeks of age. Mice were maintained and the experiments were performed according to protocols approved by the Animal Research Committee of Hirosaki University.

Gene expression studies

The total RNA was extracted from the whole aortic arch of the mice that were fed an HFC diet for 5 weeks and from the microdissected atherosclerotic lesions of the aortic arch of mice fed an HFC diet for 12 weeks. The microdissection of the atherosclerotic lesion of the aorta was performed as previously described by Khallou-Laschet et al. [18]. The extracted RNA was reverse-transcribed using a PrimeScript II First-strand cDNA Synthesis Kit (Takara, Shiga, Japan). Quantitative PCR (qPCR) was performed on a CFX100 (Bio-Rad, Hercules, CA, USA) and SYBR Premix Ex Taq (Takara) or Premix Ex Taq (Takara) with the primers and probes listed in the Supplementary Table. The expression levels of the genes of interest were normalized to the level of expression of the housekeeping gene *36B4*.

Analysis of lesion morphology

Mice were sacrificed and perfused with phosphate-buffered saline (PBS; Wako, Osaka, Japan). The entire aortic tree was obtained by removing the fat and other tissues around the vessels; the aorta was then opened longitudinally and fixed with 10% formaldehyde (Wako) in PBS. Then, the aorta was stained with oil red O (Sigma, St. Louis, MO, USA) and digitally scanned using a stereoscopic microscope with a digital camera (Olympus, Tokyo, Japan). The lesion area was assessed using Adobe Photoshop (Adobe, San Jose, CA, USA) and ImageJ (NIH, Bethesda, MD, USA) software, and the size of the lesion, as a percentage of the total aortic area, was calculated and presented as the circles represent the individual mice and the black lines indicate the mean. To analyze the aortic sinus lesions, the perfused hearts were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) in PBS for 16 h, which was followed by an incubation in 10% sucrose (Wako) for 24 h at 4 °C. The fixed hearts were embedded in OCT (Sakura Finetek, Tokyo, Japan) and frozen in liquid nitrogen. Sections (5 μm) taken at the aortic valve area were used for oil red O staining, hematoxylin and eosin (HE) staining, and immunohistochemistry. Rabbit anti-Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rat anti-MOMA-2 (Serotec, Oxford, UK) primary antibodies were used for immunohistochemistry. Alexa Fluor-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) were used to visualize Nrf2 (green, Alexa 488 anti-rabbit IgG) and MOMA-2 (red, Alexa 594 anti-rat IgG). The nuclei were counterstained with DAPI (blue) (Dojindo, Kumamoto, Japan). Sample specimens were imaged on a Leica DMI6000 B microscope (Leica Microsystems, Wetzlar, Germany) using the LAS AF6500 software (Leica) and were photographed with a Leica DFC360 FX monochrome camera or Leica DFC290 HD color camera using identical settings for all images.

BMT

At 6 weeks of age, the A0N2 or A0N0 mice were lethally irradiated (9 Gy from an X-ray source, Hitachi Co. Tokyo, Japan). After irradiation, 1×10^7 BM cells from gender-matched mice at 6 to 12 weeks of age were injected into the tail vein of the irradiated mice. Subsequently, the recipient mice were allowed to recover for 4 weeks with a normal chow diet, and then, the mice were fed an HFC diet for 12 weeks to induce atherosclerosis.

Statistical analysis

The statistical significance was determined using Student's *t test*. For the BMT experiments, the statistical significance was determined by ANOVA followed by an SNK multiple comparison test.

Results

Nrf2 gene deletion has no effect on the early stages of atherosclerotic lesion formation but provides significant protection at a later stage

The *ApoE* KO mouse is a useful animal model for the analysis of atherosclerosis formation because these animals develop atherosclerotic lesions ranging from fatty streaks to fibrous plaques [19]. To investigate the role of Nrf2 in the development of atherosclerosis in detail, we generated *ApoE* and *Nrf2* double knockout mice and analyzed their susceptibility to atherosclerosis. Oil red O staining of the aorta revealed that the A0N0 and the A0N2 (*ApoE* KO and *Nrf2* wild-type) mice fed an HFC diet for 3 or 5 weeks had similar percentages of atherosclerotic lesion area in both the male and the female mice (Fig. 1A–B and D–E). However, the A0N0

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