Ablation of Interferon Regulatory Factor 3 Protects Against Atherosclerosis in Apolipoprotein E–Deficient Mice


Abstract—The secretion of adhesion molecules by endothelial cells, as well as the subsequent infiltration of macrophages, determines the initiation and progression of atherosclerosis. Accumulating evidence suggests that IRF3 (interferon regulatory factor 3) is required for the induction of proinflammatory cytokines and for endothelial cell proliferation. However, the effect and underlying mechanism of IRF3 on atherogenesis remain unknown. Our results demonstrated a moderate-to-strong immunoreactivity effect associated with IRF3 in the endothelium and macrophasces of the atherosclerotic plaques in patients with coronary heart disease and in hyperlipidemic mice. IRF3−/−ApoE−/− mice showed significantly decreased atherosclerotic lesions in the whole aorta, aortic sinus, and brachiocephalic arteries. The bone marrow transplantation further suggested that the amelioration of atherosclerosis might be attributed to the effects of IRF3 deficiency mainly in endothelial cells, as well as in macrophages. The enhanced stability of atherosclerotic plaques in IRF3−/−ApoE−/− mice was characterized by the reduction of necrotic core size, macrophage infiltration, and lipids, which was accompanied by increased collagen and smooth muscle cell content. Furthermore, multiple proinflammatory cytokines showed a marked decrease in IRF3−/−ApoE−/− mice. Mechanistically, IRF3 deficiency suppresses the secretion of VCAM-1 (vascular cell adhesion molecule 1) and the expression of ICAM-1 (intercellular adhesion molecule 1) by directly binding to the ICAM-1 promoter, which subsequently attenuates macrophage infiltration. Thus, our study suggests that IRF3 might be a potential target for the treatment of atherosclerosis development. (Hypertension. 2017;69:510-520. DOI: 10.1161/HYPERTENSIONAHA.116.08395.)

Key Words: atherosclerosis, atherosclerotic plaque, coronary artery disease, endothelial cells, inflammation

Atherosclerosis is a chronic inflammatory disease of large and medium arteries, which depends on innate and adaptive immune responses. It is a critical pathological process underlying clinical cardiovascular events that are the predominant cause of mortality and morbidity in industrialized countries worldwide. An intact endothelium is required for maintaining normal physiological vascular function, including barrier function, thromboresistance, and anti-inflammatory effects. However, after exposure to modified low-density lipoprotein (LDL), endothelial dysfunction produces not only structural but functional alterations near curvatures, arterial branch ostia, and bifurcations, where disrupted flow with large oscillation occurs. The increased permeability of endothelial cells caused by apoptosis and the secretion of ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1) facilitate the infiltration of plasma-modulated lipoproteins and monocyte adhesion/aggregation at the subendothelium. Subsequently, the recruited monocyte-macrophages transform into lipid-laden foam cells by uptaking modified lipoproteins. Foam cells in turn secrete more proinflammatory cytokines and extracellular matrix components and promote the migration and proliferation of vascular smooth muscle cells (SMCs), which disrupts the stability of atherosclerotic plaques. The enlargement of the necrotic core caused by ineffective clearance ultimately leads to atherothrombotic vascular diseases, such as myocardial infarction, sudden cardiac death, and stroke.

The interferon regulatory factor (IRF) family comprises transcriptional regulators of the interferon-induced signaling pathway. In mammals, the IRF family includes 9 members (IRF1-9), which are involved in various biological functions,

Received August 29, 2016; first decision September 12, 2016; revision accepted December 8, 2016.

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Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYPERTENSIONAHA.116.08395

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including innate immune responses, hematopoietic development, and cell survival.\textsuperscript{14-16} In contrast with other IRFs, IRF3 is found in an inactive form in the cytoplasm of various cell types. Once phosphorylated in response to various stimuli, IRF3 translocates into the nucleus and leads to the induction of type I interferon and interferon-induced gene transcription.\textsuperscript{17-19} Previous studies have demonstrated that the LPS-MyD88 independent kinase complex phosphorylates IRF3 and results in the production of cytokines involved in innate immunity.\textsuperscript{20} In addition, IRF3 induces proatherosclerotic gene expression, including that of RANTES, Ccl5, and Cxcl10,\textsuperscript{21-23} and affects endothelial cell proliferation.\textsuperscript{24} Recently, our group has clarified that IRF3 contributes to cerebral ischemic injury,\textsuperscript{25} neointima formation,\textsuperscript{26} hepatic steatosis,\textsuperscript{27} and cardiac hypertrophy.\textsuperscript{28} However, the specific role of IRF3 in the development of atherosclerosis is unclear.

In this study, after our discovery that IRF3 is upregulated and localized in both endothelial cells and macrophages in patients with coronary heart disease (CHD) and in hyperlipidemic mice, we found that IRF3\textsuperscript{−/−}ApoE\textsuperscript{−/−} mice were resistant to high-fat diet (HFD)-induced atherogenesis. Furthermore, experiments in cross-group bone marrow transplantation indicated that IRF3 deficiency in endothelial cells was more critical for the inhibition of plaque development than its deficiency in bone marrow-derived macrophages. The effect of IRF3 deficiency in endothelial cells was contributed by the reduction of ICAM-1 and VCAM-1 secretion and macrophage infiltration. Our results demonstrated that IRF3 plays a proatherogenic role by increasing inflammation and the instability of atherosclerotic plaques.

Methods and Materials

Human Specimens

Human atheromatous plaques were collected from the coronary arteries of patients with CHD who were undergoing heart transplantation. The control samples were obtained from normal heart donors who were not suitable for transplantation for nonvascular reasons. All the procedures involving human samples were performed according to the principles outlined in the Declaration of Helsinki and were approved by the Renmin Hospital of Wuhan University Institutional Review Board in Wuhan, China.

Animals and Treatment

IRF3 global knockout mice (IRF3\textsuperscript{−/−} on a C57BL/6 background) were generously provided by T. Taniguchi.\textsuperscript{29} To obtain the IRF3\textsuperscript{−/−}ApoE\textsuperscript{−/−} mice, we generally crossbred the IRF3\textsuperscript{−/−} with ApoE\textsuperscript{−/−} to get IRF3\textsuperscript{+/−} mice. Bone marrow cells from the donor IRF3\textsuperscript{−/−}ApoE\textsuperscript{−/−} or ApoE\textsuperscript{−/−} mice were isolated and injected into ApoE\textsuperscript{−/−} and IRF3\textsuperscript{−/−}ApoE\textsuperscript{−/−} recipient mice through retro-orbital plexus. After 4 weeks of recovery, mice in all 4 groups were fed an HFD for an additional 16 weeks and then subjected to atherosclerotic lesion analysis. The DNA from a blood treatment using a TIANamp Genomic DNA Kit was applied to confirm bone marrow reconstitution via a polymerase chain reaction (PCR) analysis.

RNA Isolation and Quantitative Real-Time PCR

After being extracted using a TRizol reagent, the total mRNA from the whole aorta was collected and depurated using DNase. cDNAs were then obtained using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN). Inflammatory cytokines were identified by using quantitative real-time PCR with LightCycler 480 SYBR Green I Master Mix (Roche) and a LightCycler 480 QPCR System (Roche) in accordance with the manufacturer’s instructions. The quantified transcripts from the samples were normalized against GAPDH gene expression.

Western Blotting Analysis

Five micrograms of protein, extracted from the aorta and homogenized in a lysis buffer as described,\textsuperscript{30} was processed and then incubated with primary antibodies overnight at 4°C. After incubation with secondary antibodies, relative expression was visualized using a FluorChem E Imager (ProteinSimple, FluorChem E), and membranes were treated with enhanced chemiluminescence reagents (170-5061; Bio-Rad). The specific protein expression was normalized against GAPDH expression.

Immunofluorescence Staining

The mouse tissue sections were performed according to the protocols described earlier.\textsuperscript{31} After incubating with primary antibodies, the slice was washed in phosphate-buffered solution and incubated with the relevant secondary antibody for another hour. Images were obtained using a fluorescence microscope (OLYMPUS DX51) and DP2-BSW software (version 2.2), and the images were analyzed using Image-Pro Plus 6.0.

Adenovirus Infection and Cell Migration Assay

The AdshIRF3 was constructed as described\textsuperscript{26} and AdshRNA as a control group. For the migratory macrophage analysis, modified Boyden chambers with Costar Transwell inserts (Corning) were used. RAW 264.7 cells (5x10\textsuperscript{4}) were added to the top well, whereas human umbilical vein endothelial cells (HUVECs) were treated with oxidant LDL or TNF-α (tumor necrosis factor-α) in the bottom well for 6 hours. The cells that migrated to the bottom were fixed and stained with crystal violet/20% methanol and counted.

Statistical Analysis

All data are expressed as the mean±SD. Comparisons between groups were evaluated using a 2-tailed Student t test or 1-way ANOVAs. All statistical analyses were performed using SPSS, version 16.0. P values <0.05 were considered to be statistically significant.

Results

Upregulation of IRF3 Expression in the Atheromatous Lesions of Human and Mouse

To explore the potential role that IRF3 plays in the development of atherosclerosis, we first collected pathological coronary
arteries from patients with CHD and from ApoE (apolipoprotein E)–deficient mice, a commonly used atherosclerotic animal model, to observe whether the IRF3 expression levels were changed. Western blot analysis revealed markedly increased expression of IRF3 in patients with CHD compared with healthy individuals (Figure 1A). Significantly increased IRF3 levels were also found in atherosclerotic plaques of hyperlipidemic ApoE-deficient mice that received an HFD for 28 weeks compared with those fed normal chow (Figure 1B). The right coronary arteries of patients and the aortic roots of mice, where atherosclerotic plaques accumulate because of oscillatory shear stress, were examined via immunofluorescence staining. Double immunofluorescence staining revealed a strong immunoreactivity of IRF3 expression in the endothelial cell layer (CD31-positive cells) of atherosclerotic arteries compared with healthy arteries (Figure 1C). Similar results

Figure 1. IRF3 (interferon regulatory factor 3) is upregulated in the endothelium and macrophages of atheromatous lesions in humans and mice. A and B, Western blotting analysis of IRF3 protein levels in the right coronary arteries of humans (A) or the aortas of mice (B). The expression levels were normalized against GAPDH and quantified (n=4). C and D, Costaining of the endothelium in human coronary arteries (C) or cross sections of the aortic sinus from ApoE−/− mice (D) for IRF3 (red) and CD31 (green). Scale bar: 50 μm, n=3. E, Costaining of macrophages in patients with coronary heart disease (CHD) or cross sections of the aortic sinus in hyperlipidemic ApoE−/− mice for IRF3 (red) and MAC3 (green) Scale bar: 20 μm, n=3. F, The IRF3 expression in HUVECs or BMDMs upon oxidant LDL (Ox-LDL) treatment. *P<0.05. BMDMs indicates bone marrow–derived macrophages; HFD, high-fat diet; HUVECs, human umbilical vein endothelial cells; LDL, low-density lipoprotein; MAC3, macrophage-3 antigen; NC, normal chow; and PBS, phosphate-buffered solution.
were obtained via immunofluorescence costaining in advanced atherosclerotic lesions from the aortic roots of ApoE−/− mice in the experimental group versus the control group (Figure 1D). Moreover, IRF3 was also found to be expressed in macrophages of atheromatous plaques in patients with CHD and in hyperlipidemic ApoE−/− mice (Figure 1E). In addition, a remarkable increase in IRF3 expression was obtained in the HUVECs on oxidant LDL stimulation, whereas such increase was slight in bone marrow–derived macrophages (Figure 1F). Collectively, these data indicate that IRF3 upregulation is
Depletion of IRF3 Decreases the Development of Atherosclerosis

To determine whether the upregulation of IRF3 expression in atherosclerotic plaques contributes to atherosclerosis, we used a double knockout strategy by crossing IRF3-deleted mice with ApoE−/− mice to assess the effects of IRF3 on atherosclerosis in vivo. Western blotting verified the successful generation of the experimental mice (Figure 2A). Eight-week-old male IRF3−/−ApoE−/− mice and their ApoE−/− littermates were fed a normal chow or HFD diet for 28 weeks to stimulate atherosclerotic lesion development. An en face analysis of the whole aortas showed a significant reduction (3.42% versus 9.20%) of plaque development in IRF3−/−ApoE−/− mice compared with ApoE−/− mice when fed with normal chow (Figure 2B). In HFD group, atherosclerotic lesion area increased to 41.81% in the ApoE−/− littermates but was inhibited to 21.06% in IRF3-ablated mice (Figure 2B).

The plaque volume at aortic sinus and brachiocephalic arteries were also measured to assess the effect of IRF3 on atherogenesis in mice. A quantitative analysis showed a significant decrease in lesion area in the IRF3−/−ApoE−/− mice compared with the ApoE−/− mice, both in the aortic root (Figure 2C) and in the brachiocephalic arteries (Figure 2D). We did not observe significant differences in the plasma triglycerides, plasma cholesterol levels, or lipoprotein profiles (very low-density lipoprotein, intermediate-density lipoprotein, LDL, and high-density lipoprotein) between the genotypes fed with an HFD (Figure 2E and 2F).

IRF3 Deficiency Improved Plaque Stability

The content of vulnerable plaques is more responsible for the outcome of clinical cardiovascular complications than plaque size. IRF3−/−ApoE−/− mice showed smaller necrotic cores in their aortic sinuses and brachiocephalic arteries than did ApoE−/− mice, and the fibrous cap cover was thicker (Figure 3A). We next investigated other surrogate properties contributing to plaque stability, including collagen deposition, smooth muscle cells, macrophages, and lipid burden. First, the percentage of collagen in IRF3−/−ApoE−/− mice increased by 33% relative to the percentage in ApoE−/− littermates (Figure 3B and 3F).

Second, the SMC content of the atherosclerotic lesions showed a coordinated effect with a significant 2-fold increase in IRF3−/−ApoE−/− mice compared with controls (Figure 3C and 3F). Third, the infiltrated macrophages dramatically decreased by almost 4.6-fold in the lesions of IRF3−/−ApoE−/− mice compared with ApoE−/− mice (Figure 3D and 3F). Finally, Oil Red O staining revealed a robust 25% decrease in lipid accumulation in the lesions of IRF3−/−ApoE−/− mice compared with controls (Figure 3E and 3F). Next, we scored plaque stability as described earlier, and the result showed that IRF3 ablation remarkably increased the plaque stability (Figure 3F).

Atheroprotective Effect of IRF3 Ablation Is Mainly Contributed by Its Deficiency in Endothelial Cells

To determine which is dominant for the atheroprotective effect of IRF3 ablation between IRF3 deficiency in the arterial walls and in the hematopoietic cells, we generated lethally irradiated ApoE−/− or IRF3−/−ApoE−/− mice and transplanted bone marrow cells from IRF3−/−ApoE−/− or ApoE−/− mice into the irradiated mice. After 4 weeks of recovery, the genotypes were verified via a PCR analysis of genomic DNA isolated from the white blood cells of both receptors (Figure 4A). Four groups of chimeric mice were generated, ApoE−/− to ApoE−/−, ApoE−/− to IRF3−/−ApoE−/−, IRF3−/−ApoE−/− to ApoE−/−, and IRF3−/−ApoE−/− to IRF3−/−ApoE−/−. The chimeric mice were fed an HFD for an additional 16 weeks to induce atherogenesis. We noticed that the ApoE−/−→IRF3−/−ApoE−/− group developed 68% less lesion than ApoE−/−→ApoE−/− group (6.48% versus 20.25%). However, the IRF3−/−ApoE−/−→ApoE−/− group developed only 32% less lesion than ApoE−/−→ApoE−/− group (13.68% versus 20.25%). Plaque development was significantly slower in mice with IRF3 deficiency in aorta wall than in mice with IRF3 deficiency in bone marrow–derived hematopoietic cells (6.48% versus 13.68%; Figure 4B). Taken together, these results indicated that IRF3 deficiency in the aorta walls (mainly in endothelial cells because IRF3 is mainly expressed in endothelial cells as shown in Figure 1) is more critical than IRF3 deficiency in bone marrow–derived hematopoietic cells for atherogenesis reduction resulted from IRF3 ablation. A similar result was obtained about the lesion areas of the aortic sinus (Figure 4C).

IRF3 Deficiency Decreases Plaque Inflammation

Given that inflammation plays a vital role in atherogenesis, we examined the effect of IRF3 deficiency on the expression of inflammatory mediators. We first detected whether IRF3 deletion affects the number or subset of circulating classical monocytes. The flow cytometry analysis showed no significant difference in the numbers of monocyte between the ApoE−/− and IRF3−/−ApoE−/− group marked by CD11b+CD115. However, the IRF3−/−ApoE−/− mice showed a significant decrease in Ly6Chigh monocytes subset, whereas an increase in Ly6Chow monocytes subset (Figure 5A). The mRNA levels of proinflammatory markers, such as TNF-α, MCP-1, IL-6 (interleukin 6), and IL-1β (interleukin-1β), were decreased in the atherosclerotic lesions of IRF3−/−ApoE−/− mice compared with those of ApoE−/− mice (Figure 5B). Similarly, the IL-6, IL-1β, TNF-α, and inducible nitric oxide synthase levels in the serum were all significantly lower in IRF3−/−ApoE−/− mice than in controls (Figure 5C). Furthermore, immunofluorescent staining analyses of IL-6 and IL-10 revealed less abundance in the lesions of IRF3−/−ApoE−/− mice than in lesions of ApoE−/− mice litters (Figure 5D).

Deletion of IRF3 Attenuates Adhesion Molecule Secretion and Subsequent Macrophage Infiltration

During early atherogenesis, ICAM-1 and VCAM-1 secreted by dysfunctional endothelial cells mediate monocyte attraction and adhesion to the endothelium. The immunostaining of the aortic root cross sections with ICAM-1–specific antibodies revealed markedly decreased expression of ICAM-1 in the endothelial cell layer of IRF3−/−ApoE−/− mice compared with ApoE−/− mice (Figure 6A). In addition, a moderate decrease in expression of VCAM-1 in the endothelium was also observed in IRF3−/−ApoE−/− mice (Figure 6A). Concomitantly, IRF3 ablation significantly reduced the expression of VCAM-1 and ICAM-1 in IRF3−/−ApoE−/− mice in protein level (Figure 6B). More importantly, we found that IRF3 deletion significantly reduced the abundance of monocytes in the plaque indicated...
by Ly6C immunochemical staining (Figure 6C), suggesting the significant reduction of monocyte recruitment. To figure out whether the reduction of ICAM-1 and VCAM-1 expression after IRF3 ablation is because of direct suppression of gene transcription through an interaction with the promoter, we performed bioinformatics analysis and identified a series of putative interferon stimulated response element–binding sites in ICAM-1 promoter (designated P1–P4). To verify the potential binding sites, we performed chromatin immunoprecipitation of Myc-IRF3 in HUVECs followed by quantitative PCR of the ICAM-1 and VCAM-1 promoters. Chromatin immunoprecipitation (ChIP) assays demonstrated that the binding site for IRF3 was enriched in the P1 region but not in any of the other regions, thus indicating that P1 in ICAM-1 contains the primary site for IRF3 binding (Figure 6D), whereas no significant IRF3 binding site was observed in the VCAM-1 promoter. When treated with TNF-α, IRF3-knocking down HUVECs (Figure S1A in the online-only Data Supplement) expressed Myc–IRF3 in HUVECs followed by quantitative PCR of the ICAM-1 and VCAM-1 promoters. Chromatin immunoprecipitation (ChIP) assays demonstrated that the binding site for IRF3 was enriched in the P1 region but not in any of the other regions, thus indicating that P1 in ICAM-1 contains the primary site for IRF3 binding (Figure 6D), whereas no significant IRF3 binding site was observed in the VCAM-1 promoter.

Figure 3. IRF3 (interferon regulatory factor 3) ablation decreases necrotic area and increases plaque stability. A, Representative sections of hematoxylin and eosin (H&E)–stained aortic roots and brachiocephalic arteries from each group (* indicates necrotic area). Scale bar: 100 μm, n=6. The bar graph shows a quantification of the anuclear, afoibrotic, and eosin-negative necrotic areas. *P<0.05 compared with ApoE−/− littermates. B–E, From the top to the bottom, photographs of the atherosclerotic lesions (n=3–5) stained for collagen with picrosirius red (B), immunostained for smooth muscle cells (SMCs) with anti–smooth muscle actin (green; C), immunostained for macrophages (MACs) with anti–CD68 (red; D), or stained for lipid accumulation with Oil red O (E). F, The quantification and plaque stability score of IRF3−/−ApoE−/− mice compared with ApoE−/− mice. *P<0.05 compared with ApoE−/− littermates.
drastically reduced ICAM-1 and VCAM-1 expression in both mRNA (Figure S1B) and protein (Figure 6E) levels compared with HUVECs infected by the control virus. The Transwell migration of macrophages induced by TNF-α-stimulated HUVECs was also significantly inhibited by AdshIRF3 transfection (Figure 6F). In addition, similar results and decreased proinflammatory cytokines were obtained when these cells were stimulated by oxidant LDL (Figure S1C, S1D, S1E). These results indicated that the effect of IRF3 on atherogenesis partially depended on direct and positive regulation of ICAM-1 expression by IRF3 at transcription level.

Discussion

Our study is the first to demonstrate that IRF3 is responsible for accelerated atherogenesis in both endothelial cells and macrophages, and this effect is largely mediated by the production of adhesion molecules and inflammation, thus leading to increased necrotic cores and vulnerable plaques. From a mechanistic point of view, the ablation of IRF3 suppresses VCAM-1 expression and (especially) ICAM-1 expression by directly binding to the ICAM-1 promoter, which subsequently attenuates macrophage migration and infiltration.

Endothelial cells play a critical role in maintaining physiological vascular homeostasis and functions, including inhibition of thrombosis, barrier function, and anti-inflammatory responses. Atherosclerosis preferentially develops in curvatures, arterial branch ostia, and bifurcations, where the endothelium is exposed to turbulent blood flow and oscillatory shear stress. The stress plays an important proatherogenic role and disables the stability of atherosclerotic plaques. Endothelial dysfunction is a part of the response to the injury caused by hypercholesterolemia exposure. It is characterized by impaired endothelial repair or increased permeability, increased leucocyte adhesion, lipoprotein infiltration, and inflammation, which
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initiate the process of atherosclerosis. Understanding the mechanisms underlying the regulation of endothelial function is critical for developing effective treatment for atherosclerosis.

The present study showed that IRF3 is more abundant in the coronary arteries of patients with CHD and in the aortic roots of hyperlipidemic mice, particularly in the endothelial cells, suggesting that IRF3 may be important for endothelial homeostasis and development of atherosclerosis. Indeed, ablation of IRF3 significantly restricted the atherogenesis in HFD-fed ApoE−/− mice. In addition, the bone marrow transplantation experiment demonstrated that the IRF3 deficiency in endothelial cells but not hematopoietic cells largely accounted for the antiatherosclerotic effect. The disruption of endothelial integrity caused by the increased apoptosis of endothelial cells and the secretion of adhesion molecules, including the upregulated expression of VCAM-1 and ICAM-1 via activated

Figure 5. The reduction of inflammatory cytokine production by IRF3 (interferon regulatory factor 3) ablation. 

A, Top, Representative flow plot of mean percentage of CD11b−CD115+ monocyte in the blood of IRF3−/−ApoE−/− mice and ApoE−/− mice. Bottom, Ly6C+ and Ly6C− monocyte populations in the blood of IRF3−/−ApoE−/− mice and ApoE−/− mice. B, Serum TNF-α (tumor necrosis factor-α), MCP-1 (monocyte chemoattractant protein-1), IL-6 (interleukin 6), and IL-1β (interleukin 1β) levels were measured in ApoE−/− and IRF3−/−ApoE−/− mice via ELISA (n=6). *P<0.05 compared with their ApoE−/− littermates. C, Quantitative real-time polymerase chain reaction of the mRNAs that encode inflammation in the aorta (n=3). *P<0.05 compared with their ApoE−/− littermates. D, The immunofluorescent staining of inflammatory gene expression (both in red color) on sections from the aortic sinus in ApoE−/− and IRF3−/−ApoE−/− mice (n=3). iNOS indicates inducible nitric oxide synthase.
endothelial cells, facilitate the aggregation of circulating monocytes/macrophages into subendothelial spaces, aggravate the development of atherosclerosis, and destabilize atherosclerotic plaques. Importantly, we noticed that both the mRNA and the protein level of VCAM-1 and ICAM-1 were decreased in the aortic roots of IRF3-deficient mice and in HUVECs after knocking down IRF3 expression. The effect of IRF3 on ICAM-1 expression was mediated by directly binding to the promoter of ICAM-1, whereas not for VCAM-1. Thus, we speculated that IRF3 directly increased the ICAM-1 expression and promoted the recruitment of monocyte-macrophage infiltration, subsequently producing cytokines and chemokines to accelerate the endothelial cell injury, which can induce VCAM-1 expression. Exposure to hypercholesterolemia and the oscillatory shear stress mechanotransduction are converted into specific biochemical signals that activate intracellular signaling pathways. These pathways ultimately activate numerous endothelial genes, such as ICAM-1 and VCAM-1, that have

Figure 6. Impaired adhesion molecule secretion and macrophage infiltration under IRF3 (interferon regulatory factor 3) deficiency. A, The immunofluorescence costaining of atherosclerotic plaques with anti–ICAM-1 (intercellular adhesion molecule 1; red) or anti–VCAM-1 (vascular cell adhesion molecule 1; red) and anti–CD31 (green). Scale bar: 50 μm, n=3. B, Western blot analysis of VCAM-1 and ICAM-1 in the aortas of ApoE−/− and IRF3−/−ApoE−/− mice fed a high-fat diet (HFD) for 28 weeks (n=3). *P<0.05 vs ApoE−/−. C, Ly6C immunohistochemistry staining of cross sections from the aortic sinus to detect monocyte accumulation. D, The relative enrichment of IRF3 binding at the 4 putative interferon-stimulated response elements of ICAM-1 tested via chromatin immunoprecipitation. E, Western blot analysis of VCAM-1 and ICAM-1 expression in human umbilical vein endothelial cells (HUVECs) transfected with AdshRNA or AdshIRF3 on phosphate-buffered solution (PBS) or TNF-α (tumor necrosis factor-α) treatment. F, The number of migrated macrophages induced by the HUVECs transfected with AdshRNA or AdshIRF3 treated with PBS or TNF-α. *P<0.05 vs controls.
potentially proatherogenic properties and contain shear stress response elements in their promoter regions, which depend on or are independent of transcription factor–mediated regulation. Accordingly, we propose that the inhibition of the IRF3-ICAM-1/VCAM-1 axis should be considered to be a crucial target for the prevention of aggravated atherosclerosis.

Homing of macrophages to atherosclerotic plaques critically regulates initiation, extension, inflammation, and rupture of plaques. Our data showed that IRF3 disruption restricted oxidant LDL–induced macrophage migration by decreasing ICAM-1 and VCAM-1 expression. Published data have demonstrated that IRF3 plays a key role in the production of the cytokines implicated in innate immunity and proatherosclerotic gene expression. Our work demonstrated a dramatic decrease in inflammatory mediators in terms of mRNA, as well as serum, and protein levels in IRF3-deficient mice. In patients with clinical cardiovascular diseases, the development of atherosclerosis, especially the alteration of plaque morphology, is a more significant predictor of plaque rupture and occurrence of acute clinical events than plaque size. Necrotic core formation is a key determinant of vulnerable plaques. Our study demonstrated that IRF3 deficiency significantly decreased the size of necrotic cores both in the aortic sinus and in the brachiocephalic arteries. Furthermore, the fibrous cap cover was thicker. In addition, an analysis of the other features that contributed to vulnerable plaques in IRF3-deficient mice showed markedly increased collagen deposition and SMC content, whereas macrophage infiltration and lipid accumulation showed a significant reduction. Our results demonstrated that IRF3 is a possible candidate for the treatment of atherogenesis, particularly for maintaining plaque stability.

Most of the current literature has described IRF3 function in relation to interferon-mediated immunity. Therefore, the immunity-independent roles of IRF3 are poorly understood. Our recent research has unveiled a multifunctional role of IRF3 on different pathological stimuli, beyond its immune regulatory roles. In fact, IRF3 exerts distinct functions in different tissues and cell types via specific mechanisms. For example, IRF3 deficiency in cases of overnutrition aggravates hepatic steatosis and insulin resistance through the IKKβ/ NF-κ B (IKK-beta/nuclear factor-kappa B) signaling pathway in hepatocytes, whereas IRF3 regulates initiation, extension, inflammation, and rupture of plaques.

In our present study did not find any significant differences in the metabolic lipid profiles between IRF3−/−ApoE−/− mice and wild-type mice. The pressure overload–induced upregulation of IRF3 in cardiac myocytes attenuates the development of cardiac hypertrophy and heart failure via an interaction with ERK2 (extracellular regulated protein kinase 2) that inhibited the activation of ERK1/2 signaling. The reduction of IRF3 in vascular SMCs in response to vascular injury protects against adverse intimal hyperplasia, depending on IRF3-PPARG (peroxisome proliferator-activated receptor g) interactions. In neurons, the progression of cerebral ischemic injury is attenuated in IRF3-deficient rats. Our serial studies collectively expand this emerging field by showing the novel role of IRF3 that is independent of interferon-mediated immune responses.

In conclusion, we demonstrated that the ablation of the IRF3 associated with endothelial cells and macrophages inhibits the development of atherogenesis and enhances plaque stability by contributing to the inhibition of adhesion molecule secretion and vascular inflammation. Our work highlights the important role of neutralizing IRF3 as a potential pharmacological therapeutic approach to prevent atherosclerosis and stabilize atherosclerotic plaques.

Perspectives

The in vivo and vitro experiments suggest that IRF3, a member of the interferon transcription factor family, functions as a positive regulator of the atherosclerosis partially through directly binding with the ICAM-1 promoter, and thereby accumulate macrophage infiltration, which subsequently promote the inflammation response. These observations indicate that blockade of IRF3 expression/activity would be beneficial for the prevention of atherosclerosis development and coronary artery diseases.

Acknowledgments

We thank T. Taniguchi for providing IRF3 knockout mice. We acknowledge the valuable technological assistance that Cheng Du, Qiao-Fang Wei, Meng-lin Chao, and Ling Yang provided for this study.

Sources of Funding

This work was supported by grants from the National Science Fund for Distinguished Young Scholars (No 81425005), the Key Project of the National Natural Science Foundation (No 81330005), the National Natural Science Foundation of China (No 81370209; No 81370365; No 81270184; No 31371481), National Science and Technology Support Project (Nos 2013YQ030293-05, 2014BAI02B01, 2015BAI08B01, and 2016YFF0101500).

Disclosures

None.

References

The current study advances our understanding about the role of IRF3 in the atherogenesis and provides a novel target for the intervention of atherosclerosis and coronary diseases.

**Novelty and Significance**

**What Is New?**

- IRF3 (interferon regulatory factor 3) is first identified to be relevant to both human and mouse atherosclerotic plaque.
- IRF3 is a key modulator responsible for the development of atherosclerosis by increasing the adhesion molecules secretion partially by directly binding to the ICAM-1 (intercellular adhesion molecule 1) promoter.
- The current study advances our understanding about the role of IRF3 in the atherogenesis and provides a novel target for the intervention of atherosclerosis and coronary diseases.

**What Is Relevant?**

- The important functions of IRF3 beyond immune response have been identified in hepatic steatosis, cardiac hypertrophy, intimal hyperplasia, and cerebral ischemic injury, whereas its effect on the development of atherosclerosis and its underlying mechanisms are unknown.

**Summary**

This study demonstrates that IRF3 is responsible for exaggerated atherosclerotic plaques, which is largely mediated by the production of adhesion molecules and inflammation, by directly binding to the ICAM-1 promoter.
Ablation of Interferon Regulatory Factor 3 Protects Against Atherosclerosis in Apolipoprotein E–Deficient Mice

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Hypertension. 2017;69:510-520; originally published online January 23, 2017;
doi: 10.1161/HYPERTENSIONAHA.116.08395

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Ablation of Interferon Regulatory Factor 3 protects against atherosclerosis in apolipoprotein E-deficient mice

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Supplementary Figures

Figure S1 The reduction of ICAM-1, VCAM-1 secretion and infiltrated macrophage in the absence of IRF3. A. The IRF3 expression in the HUVECs transfected with AdshRNA and AdshIRF3. B. mRNA expression level of ICAM-1 and VCAM-1 in HUVECs upon TNF-α stimulation. C. Western blot analysis of VCAM-1 and ICAM-1 in HUVECs treated with Ox-LDL. D. The number of migrated macrophages induced by the HUVECs of AdshRNA or AdshIRF3 treated with oxLDL for 24 hours. E. mRNA expression level of proinflammatory cytokines in HUVECs upon Ox-LDL stimulation.* P<0.05 versus AdshRNA group.