Fortilin reduces apoptosis in macrophages and promotes atherosclerosis

Decha Pinkaew,1 Rachel J. Le,1 Yanjie Chen,1 Mahmoud Eltorky,3 Ba-Bie Teng,4 and Ken Fujise1,2

1Division of Cardiology, Department of Internal Medicine, University of Texas Medical Branch, Galveston, Texas; 2Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, Texas; 3Department of Pathology, University of Texas Medical Branch, Galveston, Texas; and 4Institute of Molecular Medicine for the Prevention of Human Diseases, University of Texas Health Science Center, Houston, Texas

Submitted 29 July 2013; accepted in final form 9 September 2013

Pinkaew D, Le RJ, Chen Y, Eltorky M, Teng B, Fujise K. Fortilin reduces apoptosis in macrophages and promotes atherosclerosis. Am J Physiol Heart Circ Physiol 305: H1519–H1529, 2013.—Atherosclerosis, a deadly disease insufficiently addressed by cholesterol-lowering drugs, needs new therapeutic strategies. Fortilin, a 172-amino acid multifunctional polypeptide, binds p53 and blocks its transcriptional activation of Bax, thereby exerting potent antiapoptotic activity. Although fortin-overexpressing mice reportedly exhibit hypertension and accelerated atherosclerosis, it remains unknown if fortin, not hypertension, facilitates atherosclerosis. Our objective was to test the hypothesis that fortin in and of itself facilitates atherosclerosis by protecting macrophages against apoptosis. We generated fortin-deficient (fortilin−/−) mice and wild-type counterparts (fortilin+/+) on a LDL receptor (Ldlr−/−) apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 (Apobec1)−/− hypercholesterolemic genetic background, incubated them for 10 mo on a normal chow diet, and assessed the degree and extent of atherosclerosis. Despite similar blood pressure and lipid profiles, fortin−/− mice exhibited significantly less atherosclerosis in their aortae than their fortin+/+ littermate controls. Quantitative immunostaining and flow cytometry analyses showed that the atherosclerotic lesions of fortin−/− mice contained fewer macrophages than those of fortin+/+ mice. In addition, there were more apoptotic cells in the intima of fortin−/− mice than in the intima of fortin+/+ mice. Furthermore, peritoneal macrophages from fortin−/− mice expressed more Bax and underwent increased apoptosis, both at the baseline level and in response to oxidized LDL. Finally, hypercholesterolemic sera from Ldlr−/− Apobec1−/− mice induced fortin in peritoneal macrophages more robustly than sera from control mice. In conclusion, fortin, induced in the proatherosclerotic microenvironment in macrophages, protects macrophages against Bax-induced apoptosis, allows them to propagate, and accelerates atherosclerosis. Anti-fortin therapy thus may represent a promising next generation antiatherosclerotic therapeutic strategy.

atherosclerosis; fortin; macrophages; apoptosis

Atherosclerosis now is one of the most prominent global health problems (37a). After the negative outcomes of several then-promising antiatherosclerotic strategies, such as cholesteryl ester transfer protein inhibition [torcetrapib (5)], activation of the peroxisome proliferator-activator receptor [rosiglitazone (40)], and cannabinoid type-1 receptor inhibition [rimonabant (32)], a critical barrier to eliminating atherosclerosis and its complications lies in the lack of novel molecular targets, the successful targeting of which might ameliorate atherosclerosis (35, 46).

Macrophages play a key role in the development of atherosclerosis. In the early stages of atherosclerosis, LDLs in the plasma cross the endothelial barrier and enter the intima, where they become oxidized (36). Oxidized LDL (oxLDL) is not only a potent chemoattractant that recruits circulating monocytes to the intima and promotes their differentiation into macrophages but is also an inducer of macrophage apoptosis (47). When their apoptosis is inhibited by antiapoptotic proteins or inflammatory cytokines (18), macrophages survive, become activated, and avidly take up oxLDL through scavenger receptors (43). Progressive cytoplasmic accumulation of oxLDL by activated macrophages creates a characteristic “soap bubble” appearance, thereby giving them the name “foam cells.” These foam cells proliferate in response to mitogens in the microenvironment (36, 37) and secrete a number of proinflammatory cytokines and chemokines (12), which, in turn, attract more monocytes to the intima, augmenting the inflammatory response in a vicious cycle of atherosclerogenesis.

Fortilin, also known as translationally controlled tumor protein, is a 172-amino acid nuclear-cytosolic shuttle protein that was originally cloned in 1989 by Gross and others (16) as a protein abundantly expressed in tumor cells. Fortilin, a multifunctional protein that has been implicated in various cellular functions (1, 15, 21, 22, 28, 48), possesses potent antiapoptotic activity (13–15, 25, 26, 33, 44, 49). More recently, we have shown that fortin binds the sequence-specific DNA binding domain of p53 and prevents p53 from transcriptionally activating the proapoptotic gene Bax (8). In addition, fortin binds to and stabilizes myeloid cell leukemia protein-1 (49), a macrophage survival factor (30, 41). However, the precise role of fortin in atherosclerosis has never been experimentally tested.

Given that macrophage survival is linked to the progression of atherosclerosis (10, 27), we hypothesized that fortin promotes atherosclerosis by blocking Bax-mediated apoptosis in macrophages, allowing macrophages to survive, propagate, and facilitate atherosclerogenesis.

To more definitively evaluate the role of fortin in the development of atherosclerosis in a loss-of-function system, we first attempted to generate constitutional fortin knockout mice through targeted disruption of the endogenous fortin gene (25). Mice that retained one functional copy of the fortin gene (fortin+/− mice) were found to be grossly normal and fertile, whereas mice in which both copies of the gene were disrupted (fortin−/− mice) died on embryonic day 3.5, owing...
to massive and generalized apoptosis, a phenotype that has also been reported by other investigators (7, 19). Meanwhile, the mice lacking both LDL receptor (Ldlr) and the apolipoprotein B mRNA editing enzyme catalytic polypeptide 1 (ApoB1, namely Ldlr\(^{-/-}\)ApoB1\(^{-/-}\)) mice, have emerged as a robust mouse model of human atherosclerosis, and their lipid profile faithfully mirrors that of human familial hypercholesterolemia (34).

We crossed fortilin\(^{+/+}\) mice with Ldlr\(^{-/-}\)ApoB1\(^{-/-}\) mice and generated fortilin\(^{+/+}\)Ldlr\(^{-/-}\)ApoB1\(^{-/-}\) and fortilin\(^{+/+}\)Ldlr\(^{-/-}\)ApoB1\(^{-/-}\) mice. To our surprise, the blood pressure of these two groups of mice was identical, providing us with a unique opportunity to definitively evaluate the role of fortilin in atherosclerosogenesis, separate from the influence from hypertension induced by overexpression of fortilin (9, 23).

Here, we report that a heterozygous deficiency of fortilin (fortilin\(^{+/+}\)) in a background of hypercholesterolemia facilitates the apoptosis of macrophages and ameliorates the development of atherosclerotic plaques in an animal model that exhibits similar atherosclerotic characteristics as in humans. We propose that fortilin is a proatherosclerotic molecule in and of itself that represents a viable target molecule for antiatherosclerotic therapy.

**MATERIALS AND METHODS**

**Animals.** All animal procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee in accordance with National Institutes of Health (NIH) guidelines and the “Position of the American Heart Association on Research Animal Use.” Ldlr\(^{-/-}\)ApoB1\(^{-/-}\) mice were used as a model of in vivo atherosclerosis in the presence of hypercholesterolemia. Ldlr\(^{-/-}\)ApoB1\(^{-/-}\) mice were originally generated by crossing Ldlr\(^{-/-}\) mice (Jackson Lab, Bar Harbor, ME) and ApoB1\(^{-/-}\) mice (31c); these double-knockout mice have previously been characterized (11, 34). Genotyping of Ldlr\(^{-/-}\)ApoB1\(^{-/-}\) mice was performed on tail-derived genomic DNA using standard PCR-based methods. ApoB1 was assessed with the following primer sets: 5'-TGTAGTAGTGGTGGTGGTAAAG-3' and 5'-CGAAATCTCCACGAGTAC-3'. ApoB1\(^{-/-}\) and ApoB1\(^{-/-}\) mice yield a 475-bp amplified fragment, whereas ApoB1\(^{-/-}\) mice yield no fragments.

Ldlr was assessed with the following primer sets: 5'-ACCCCCAAGCTGCTCCTCAGATGA-3' and 5'-CGAGTGTTCCTCTATCTTG-3'. Ldlr\(^{-/-}\) mice yield a 383-bp fragment, whereas Ldlr\(^{-/-}\) mice yield no fragments. To determine fortilin expression in atherosclerotic aorta, 25 Ldlr\(^{-/-}\)ApoB1\(^{-/-}\) mice aged 8 wk old were killed on normal rodent chow (Lab Diet, Richmond, VA) and euthanized at 10, 20, 30, 40, or 50 wk of age. At each time point, five animals were euthanized, and normal rodent chow (Lab Diet) and housed individually in an air-conditioned room with a 12:12-h light-dark cycle and with access to food and water. Animals were euthanized at 10 mo of age. At the time of death, animals were weighed, and blood samples from the heart were collected into microcentrifuge tubes containing EDTA. The entire aorta of each animal was excised en bloc. The very proximal portion of the ascending aorta containing the aortic valves was embedded in Tissue-Tek OCT compound (Sakura-Finetech, Torrance, CA), subjected to frozen sectioning, and used for cross-sectional atherosclerosis analysis and immunostaining. The rest of the aorta was used for en face atherosclerosis analysis as described below.

For the measurement of mouse blood pressure, the CODA mouse tail-cuff blood pressure system was used (Kent Scientific, Torrington, CT) according to the manufacturer’s instructions. We placed the mouse in a holder, which was then placed onto the warming platform. The mouse was allowed to acclimate to the holder for 5 min. The tail was then threaded through the occlusion cuff and VPR cuff, both of which were connected to the CODA controller. Twenty cycles of blood pressure measurements were performed per mouse.

For DNA fragmentation assays on oxLDL-challenged peritoneal macrophages, resident macrophages from 9-mo-old male fortilin\(^{+/+}\)Ldlr\(^{-/-}\)ApoB1\(^{-/-}\) and fortilin\(^{+/+}\)Ldlr\(^{-/-}\)ApoB1\(^{-/-}\) mice were harvested by peritoneal lavage with ice-cold PBS under sterile conditions, as previously described (48a).

**Analysis of atherosclerotic lesions.** Aortae and hearts of the mice were excised en bloc. The extent and degree of atherosclerosis were quantified by en face analysis of atherosclerosis lesions and intimal areas of the ascending aorta at the level of the aortic valve leaflets, as previously described in detail (9a, 42a). Briefly, for en face analysis of atherosclerotic lesions, the distal portion of the ascending aorta, the aortic arches, and the descending aorta down to the iliac bifurcations were pinned flat on a white wax surface, fixed with 10% (vol/vol) buffered formalin solution overnight, stained with freshly prepared and filtered oil red O solution for 1 h, rinsed twice with 78% methanol, mounted and dried on glass slides, and scanned in TIF format using the ScanScope slide scanning system (Nikon, Melville, NY). The planimetry of the entire surface area and oil red O-positive atherosclerotic lesion areas was performed on the scanned images using Sigma Scan Pro software (SPSS, Chicago, IL). For cross-sectional analysis of atherosclerosis, the roots of the ascending aorta containing the aortic valves were embedded in Tissue-Tek OCT compound (Sakura-Finetech), cryostat sections (5 μm) were obtained at the levels of aortic valve leaflets and subject to hematoxylin and eosin (H&E) staining, TUNEL assay, and other immunohistochemical staining as described below. Images of the aortae were digitally captured and stored in TIF format using the ScanScope slide scanning system (Nikon). Quantification of the atherosclerotic area was performed by ImageJ software (NIH, Bethesda, MD) and expressed as lesion areas (in mm\(^2\)).

**Western blot analysis.** Western blot analysis was performed as previously described (13–15, 26, 49) using anti-fortilin (MBL, Woburn, MA) and anti-GAPDH clone 6C5 ( Fitzgerald Industries, Acton, MA) antibodies with the following modifications. Whole aortae were cut and homogenized directly in SDS loading buffer; genomic DNA in the homogenate was sheared by sonication the sample three times. Thirty microliters of each sample were loaded in each lane of a 12% SDS-polyacrylamide gel. IRDye 800CW-conjugated secondary antibodies (LI-COR, Lincoln, NE) were used to detect bound primary antibodies. Signal intensities of the fortilin and GAPDH bands were captured and quantified using the Odyssey Infrared Imaging System (LI-COR). Relative fortilin levels were determined as the ratio of fortilin band intensity to GAPDH band intensity.

**Immunohistochemistry.** All human tissue samples were provided by the Department of Pathology of the University of Texas Medical Branch without any personal identifying information. These samples, which exhibited various degrees of atherosclerosis, were originally acquired from lower extremity arteries and had been formalin fixed and paraffin embedded. Fortilin immunostaining was performed as
previously described (26) using rabbit anti-fortilin polyclonal antibody (MBL). For mouse tissue, cryosections of the aortae were stained using anti-fortilin (MBL), anti-smooth muscle cell α-actin (Abcam, Cambridge, MA) (27a), anti-macrophage surface glycoproteins (clone F4/80, Abcam) (10a), and anti-Ki67 (clone TEC-3, DAKO, Carpinteria, CA) (31b) antibodies using 3,3′-diaminobenzidine (DAB) as the chromogen, as previously described (26). All sections were digitally imaged and stored in TIFF format using a Nikon Eclipse TS100 microscope equipped with a DS-Fi1 camera (Nikon). For TUNEL and Ki67 staining, DAB-positive cells were manually counted, and the percentage of DAB-positive cells versus total counted cells (percent TUNEL-positive cells and percent Ki67-positive cells) and the number of DAB-positive cells per unit lesion area (in mm²; an arbitrary unit) were calculated. For macrophage and smooth muscle cell staining, DAB-positive areas (in mm²), both per section and per unit lesion area, were calculated using NIS-Elements BR 3.0 software (Nikon).

Immunofluorescence and confocal microscopic analysis of fortilin and macrophages. Immunofluorescence localization experiments were performed with formalin-fixed cryosections of aortae isolated from Ldlr<sup>−/−</sup> Apobec1<sup>−/−</sup> mice. For colocalization of fortilin and macrophage cells, cryosections were fixed in 4% buffered formalin solution for 15 min at 37°C. After three washes with PBS, sections were permeabilized at room temperature with 0.2% Triton X-100 for 5 min, washed, and then incubated for 60 min in blocking solution containing 10% normal goat serum. Sections were then incubated with anti-fortilin (1:50 dilution, MBL) and F4/80 anti-macrophage surface glycoprotein (1:200 dilution, Abcam) antibodies for 2 h at room temperature followed by secondary antibodies labeled with Alexa fluor 488 and Alexa fluor 568 (Molecular Probes-Invitrogen, Carlsbad, CA) for 30 min. Sections were then washed, counterstained with 4′,6-diamidino-2-phenylindole (Sigma, St. Louis, MO), and rinsed in PBS. Coverslips were mounted on a glass slide and digitally imaged with an Olympus Fluoview1000 confocal microscope in the upright configuration with a BX61 microscope.

Real-time quantitative RT-PCR. Real-time quantitative RT-PCR was performed according to previously described methods (31a) with the following modifications. Total RNA was extracted from aortae using TRI-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. The following primers were used for the detection of mouse fortilin: forward 5′-TCCGA-CATCTACAAGATCCGG-3′ and reverse 5′-ATCTTGCCCTCCACTCTC-3′. The following probe labeled with carboxyfluorescein (FAM; Integrated DNA Technologies, IDT, Coralville, IA) and Iowa black FQ (IabkFQ; Integrated DNA Technologies) was also used: 5′-FAM-AGATCCGGCAGGCTGTGC-IAbkFQ-3′. The following primers were used for the detection of mouse GAPDH: forward 5′-TGTATGGGTTGAAACCCAGAGA-3′ and reverse 5′-GAGCCCTCCAAAATGCAAGTT-3′. The following probe labeled with 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein (JOEN; Integrated DNA Technologies) and Iowa black RQ-Sp (IabkRQSP; Integrated DNA Technologies) was also used: 5′-JOEN-ATTGATCTCCGTCAACACCACTCTGTT-IabkRQSP-3′.

Quantitative RT-PCRs were performed in quadruplicate using the TaqMan RT-PCR kit (Applied Biosystems, Carlsbad, CA) in the StepOnePlus Sequence Detector system. The critical threshold of both fortilin and GAPDH were determined from a single well. The fortilin transcript copy number from a well was normalized to the GAPDH copy number from the same well and expressed as the fold change in fortilin mRNA levels.

DNA fragmentation assay. Fragmentation of histone-associated DNA was analyzed as previously described (25) using the Cell Death Detection ELISA-Plus kit (Roche, Indianapolis, IN) according to the manufacturer’s instructions. Cell lysate (20 μl from either untreated cells or cells challenged by oxLDL and 80 μl of assay buffer containing anti-histone-biotin and anti-DNA-peroxidase were dispensed into streptavidin-coated wells of a 96-well plate in quadrupli-
cate. After an incubation and washes, captured nucleosomes were detected by 2′,3′-azido-di3-ethyl-benzthiazoline-6-sulfonic acid). Signal intensity was measured at 405 nm with a reference wavelength of 490 nm, and the signal intensity was designated as the DNA fragmentation index.

Flow cytometric analysis. Dissected and cleaned aortae from fortilin<sup>−/−</sup> Ldlr<sup>−/−</sup> Apobec1<sup>−/−</sup> and fortilin<sup>+/−</sup>Ldlr<sup>−/−</sup> Apobec1<sup>−/−</sup> mice (3 animals/group) were pooled, weighed, microdissected into small pieces, and subjected to enzymatic digestion for 60 min at 37°C with a cocktail of collagenase type XI, collagenase type I, hyaluronidase type Is, and DNase I in RPMI medium 1640 (Sigma-Aldrich). The digested tissue was filtered through a 70-μm cell strainer. Leukocytes were cleared of cell debris using OptiPrep density gradient medium (Sigma-Aldrich) according to Graziani-Bowering’s methods (15a). Cells were then stained with anti-CD11b-phycocerythrin (macrophages), anti-CD3e-allophycocyanin (T cells), and anti-CD19-allo-
phycocyanin (B cells) antibodies as well as with peridinin chlorophyll protein-Cy 5.5 annexin V and 7-amino-actinomycin D before being subjected to flow cytometry (BD FACSAria). Data were analyzed using FlowJo (version 10, Ashland, OR).

Statistical analyses. Statistical analyses were performed using Minitab statistical software (version 15, Minitab, State College, PA). The degree of spread of the data was expressed by the (±)SD. Student’s t-test was used to compare two sets of data, whereas Fisher’s ANOVA was used to compare three or more sets of data. P values of <0.05 were considered to be statistically significant.

RESULTS

Expression of fortilin protein increases during the progression of atherosclerosis. The immunostaining of noncalcified human atherosclerotic tissue showed that fortilin levels increased as atherosclerosis progressed from the fatty streak to the fibrous cap (Fig. 1A). Aortae from Ldlr<sup>−/−</sup> Apobec1<sup>−/−</sup> mice (11, 29) fed normal chow were then harvested at 10, 20, 30, 40, and 50 wk of age and subjected to H&E, anti-fortilin, and anti-F4/80 macrophage staining. The atherosclerotic lesion cross-sectional area at the level of the aortic valve increased from 0.00 to 0.15 mm² over the 50-wk period [Fig. 1, A and C (total lesion area)]. Increasing age of the mice showed a positive correlation with the extent of atherosclerosis, fortilin protein expression levels, and macrophage infiltration. The areas occupied by fortilin-expressing cells [Fig. 1, B (α-fortilin) and C (fortilin)] and macrophages [Fig. 1, B (α-MΦ) and C (MΦ)] also increased significantly with the progression of atherosclerosis. The areas of fortilin and macrophage immunoreactivity substantively overlapped (Fig. 1B, α-fortilin and α-MΦ).

Fortilin deficiency results in a lesser degree of atherosclerosis in hypercholesterolemic mice. Next, we crossed fortilin<sup>+/−</sup> mice with Ldlr<sup>−/−</sup> Apobec1<sup>−/−</sup> mice to generate fortilin<sup>+/−</sup>Ldlr<sup>−/−</sup> Apobec1<sup>−/−</sup> and fortilin<sup>+/−</sup>Ldlr<sup>−/−</sup> Apobec1<sup>−/−</sup> mice (Fig. 2A). The aortae of fortilin<sup>+/−</sup>Ldlr<sup>−/−</sup> Apobec1<sup>−/−</sup> mice showed 30% less fortilin mRNA (Fig. 2B) and 28% less fortilin protein (Fig. 2C) than fortilin<sup>+/−</sup>Ldlr<sup>−/−</sup> Apobec1<sup>−/−</sup> mice. Fortilin immunostaining of the ascending aortae of these animals confirmed that fortilin protein within the intima of the aorta was significantly more abundant in fortilin<sup>+/−</sup>Ldlr<sup>−/−</sup> Apobec1<sup>−/−</sup> mice than in fortilin<sup>+/−</sup>Ldlr<sup>−/−</sup> Apobec1<sup>−/−</sup> mice (Fig. 2D). There were no statistically significant differences in either systolic or diastolic blood pressures of the two mouse strains (Fig. 2E). These mice (male, n = 15 for each strain) were maintained on a normal chow diet and euthanized at 10 mo of age. At the time of death, there were no significant differences in
body weight, total cholesterol, triglycerides, phospholipids, and nonesterified fatty acids between fortilin+/+ Ldlr−/− Apobec1−/− and fortilin+/+ Ldlr−/− Apobec1+/− mice (Fig. 2, F and G). In this system, both the en face and cross-sectional analysis showed that fortilin+/+ Ldlr−/− Apobec1−/− mice had a significantly larger atherosclerotic burden than did fortilin+/+ Ldlr−/− Apobec1+/− mice (Fig. 3, A and B, 25–27% decrease in fortilin+/− mice).

Fortilin deficiency is associated with fewer macrophages in atherosclerotic plaques. Given that fortilin is known to possess antiapoptotic activity (13–15, 25, 26, 33, 44, 49), we evaluated apoptosis in atherosclerotic lesions by performing TUNEL staining. This approach revealed that atherosclerotic lesions from fortilin+/+ Ldlr−/− Apobec1−/− mice contained significantly more apoptotic cells than those from fortilin+/+ Ldlr−/− Apobec1+/− mice (Fig. 4A), suggesting that fortilin deficiency promotes apoptosis in these lesions.

Next, we assessed how fortilin deficiency affected the degree of infiltration of macrophages and vascular smooth muscle cells into atherosclerotic lesions. Anti-F4/80 macrophage immunostaining showed that atherosclerotic lesions from fortilin+/+ Ldlr−/− Apobec1−/− mice contained significantly more macrophages than those from fortilin+/+ Ldlr−/− Apobec1+/− mice (Fig. 4B). In contrast, anti-smooth muscle α-actin staining revealed no differences in the number of vascular smooth muscle cells between fortilin+/+ Ldlr−/− Apobec1−/− and fortilin+/+ Ldlr−/− Apobec1+/− mice (Fig. 4C), suggesting that fortilin deficiency leads to a reduction in macrophages but not vascular smooth muscle cells. Confocal microscopic analysis using anti-fortilin and anti-macrophage antibodies showed that macrophage and fortilin signals colocalized in the cellular portion of the intimal atherosclerotic lesion (Fig. 4D, white arrowheads), suggesting that fortilin deficiency leads to more macrophage apoptosis and less macrophage infiltration into atherosclerotic lesions.

Fortilin deficiency causes more macrophages to apoptose in atherosclerotic plaques. To trace the fate of macrophages in atherosclerotic plaques, we performed multicolor flow cytometric analyses on cells isolated from pooled aortae of fortilin+/+ Ldlr−/− Apobec1−/− and fortilin+/+ Ldlr−/− Apobec1+/− mice. Consistent with the data shown in Fig. 4B, per the same mass of the aorta, significantly fewer macrophages were present in the aortae of fortilin+/+ Ldlr−/− Apobec1−/− and fortilin+/+ Ldlr−/− Apobec1+/− mice than those of their wild-type fortilin+/+ Ldlr−/− Apobec1−/− counterparts (Fig. 5, A and B). More macrophages were dead (Fig. 5, C and D) due to apoptosis (Fig. 5, E and F) in the aortae of fortilin-deficient mice, suggesting that fortilin deficiency leads to more macrophage apoptosis in atherosclerotic lesions and to lesser
number of macrophages there. We did not have a sufficient number of macrophages from 10-mo-old aortae to evaluate the status of macrophage polarization.

Increase in macrophage apoptosis in atherosclerotic lesions is mediated by bax and precipitated by oxLDL. To investigate why fortilin deficiency leads to more macrophage apoptosis in atherosclerotic lesions, we turned to peritoneal macrophages induced and harvested from fortilin+/− Ldlr−/− Apobec1−/− and fortilin+/− Ldlr−/− Apobec1−/− mice. As expected, peritoneal macrophages isolated from fortilin+/− Ldlr−/− Apobec1−/− mice contained 33% less fortilin mRNA (Fig. 6A) and apoptosed more without provocation (Fig. 6B). Strikingly, in this system, the Bax gene was expressed more in macrophages from fortilin-deficient mice (Fig. 6C). Since there were no statistically significant differences in p53 protein levels between the two strains (Fig. 6D), these data suggest that the lack of fortilin [a p53 inhibitor (8)], not the increased expression of p53, led to the higher Bax gene expression in macrophages from fortilin-deficient mice. This observation in peritoneal macrophages held true for cells from the atherosclerotic aorta: Bax expression was found to be more abundant in cells from aortae of fortilin+/− Ldlr−/− Apobec1−/− mice than in those from fortilin+/− Ldlr−/− Apobec1−/− mice (Fig. 6E).

Since oxLDL has been implicated in macrophage apoptosis in an atherosclerotic milieu (24, 39), we tested the effect of oxLDL on peritoneal macrophages from fortilin+/− Ldlr−/−
Fig. 3. Fortilin deficiency ameliorates atherosclerosis in mice on the hypercholesterolemic genetic background. A: representative images of oil red O staining of aortae of fortin+/ Apobec1−/− and fortin+/ Apobec1−/− Apobec1−/− mice. n = 15 from each of the two genotypes. B: H&E staining of aortae of fortin+/ Apobec1−/− and fortin+/ Apobec1−/− mice. n = 15 from each of the two genotypes. *P < 0.05; ***P < 0.001.

Apobec1−/− and fortin+/−Ldlr−/−Apobec1−/− mice. We found that oxLDL challenge caused macrophages from fortin+/−Ldlr−/−Apobec1−/− mice to undergo more apoptosis than macrophages from fortin+/−Ldlr−/−Apobec1−/− mice (Fig. 6F).

Hypercholesterolemic sera and macrophage colony-stimulating factor induce fortin in macrophages. To investigate how fortin is induced in the atherosclerotic microenvironment, we incubated peritoneal macrophages from wild-type C57BL/6J mice with PBS or 10% sera from either wild-type C57BL/6J mice (total cholesterol: ∼550 mg/dl) or Ldlr−/−Apobec1−/− mice on the C57BL/6J genetic background (total cholesterol: ∼550 mg/dl). Fortin mRNA increased by 109% in the presence of sera from Ldlr−/−Apobec1−/− mice (Fig. 6G) compared with sera from wild-type C57BL/6J mice. We then tested whether macrophage colony-stimulating factor (M-CSF), a cytokine that is found elevated in patients with coronary artery disease (38), induces fortin. As shown in Fig. 6H, fortin mRNA levels were 1.52- and 1.66-fold higher than those at baseline compared with stimulation by 1 and 10 ng/ml M-CSF, respectively.

DISCUSSION

To the best of our knowledge, the present study is the first to investigate the direct role of fortin in atherosclerogenesis and to demonstrate a novel mechanism in which fortin facilitates atherosclerosis by protecting macrophages against apoptosis.

Our work is distinct from that of Cho and others (23). The investigators generated transgenic mice overexpressing fortin (23), crossed them with apolipoprotein E (ApoE)−/− mice to produce fortin transgenic mice on the ApoE−/− hypercholesterolemic genetic background (fortin+/ApoE−/−), placed them on a lipid-enriched Western diet for 16 wk, and evaluated the degree and extent of atherosclerosis (9). Control mice were ApoE−/− mice without fortin transgene expression (fortin+/ApoE−/−). They found that fortin+/ApoE−/− had significantly more extensive atherosclerotic lesions than fortin+/ApoE−/− mice. Unfortunately, the blood pressure of fortin+/ mice was significantly higher (∼20 mmHg) than that of fortin+/ mice (23). Since hypertension facilitates atherosclerosis (5a), it remained unclear whether 1) fortin or 2) hypertension associated with fortin overexpression accelerated atherosclerosis. The role of fortin in the development of atherosclerosis was not clearly defined in their work. In addition, the clinical relevance of the phenotypes of mice with supraphysiologically high fortin expression remained unclear. Finally, macrophages in atherosclerotic lesions were not quantified in their work. There have been no other reports on the role of fortin in atherosclerosis.

The overarching hypothesis of the present work, supported by the observation that fortin protein is abundantly expressed in atherosclerotic plaques and its expression positively correlates with the degree of atherosclerosis (Fig. 1), is that fortin facilitates atherosclerosis and that fortin is a viable molecular target of antiatherosclerosis therapy. The testing of the hypothesis was made possible by the availability of fortin+/ (fortin deficient) and fortin+/+ (fortin wild type) mice on the Ldlr−/−Apobec1−/− hypercholesterolemic genetic background whose blood pressure did not differ from each other (2). Again, this was an unexpected finding since fortin+/ mice exhibited significantly higher blood pressure (∼20 mmHg) than their fortin+/ counterparts (23), preventing the investigators from concluding that fortin, not hypertension, facilitates atherosclerosis. On the contrary, the data presented here clearly show that fortin facilitates atherosclerosis (Fig. 3).

Several mouse models of human atherosclerosis are available, but Ldlr−/−Apobec1−/− mice, which lack both Apobec1 and LDLRs, were considered the most appropriate for the present study. Ldlr−/− mice lack LDLRs, exhibit only modest hypercholesterolemia, and do not develop considerable atherosclerotic lesions when maintained on a normal diet (6, 20, 34).
In contrast, Ldlr<sup>−/−</sup> Apobec1<sup>−/−</sup> mice have markedly increased plasma cholesterol levels when maintained on a normal diet and develop extensive lesions, ranging from fatty streaks to fibrous plaques, throughout the aorta. Furthermore, in Ldlr<sup>−/−</sup> Apobec1<sup>−/−</sup> mice, most of cholesterol in plasma resides in LDL, not in chylomicrons or very-low-density lipoprotein (VLDL). Thus, this phenotype resembles the pathophysiology of human familial hypercholesterolemia as previously reported (11, 29, 34). Another animal model is the ApoE<sup>−/−</sup> mouse, which lacks ApoE, a ligand for receptors in the liver that clear remnants of chylomicrons and VLDL from circulation. The lack of ApoE leads to the accumulation in plasma of cholesterol-rich remnants (i.e., chylomicrons and VLDL) but not LDL. ApoE<sup>−/−</sup> mice exhibit high plasma cholesterol levels and robust atherosclerotic lesions when maintained on a normal diet (50). However, macrophages within atherosclerotic lesions of these mice fail to produce the antiatherosclerotic protein ApoE, thereby making the interpretation of macrophage-related data more complex than Ldlr<sup>−/−</sup> Apobec1<sup>−/−</sup> mice (4, 34).

Mechanistically, fortillin induced in macrophages by the factors found in the atherosclerotic microenvironment such as hypercholesterolemia (Fig. 6G) and inflammatory cytokines (Fig. 6H) I) protected macrophages from apoptosis (Figs. 5 and 6B) induced by oxLDL (Fig. 6F) and mediated by Bax (Fig. 6, C and E and 2) allowed macrophages to increase in number within atherosclerotic lesions (Figs. 4B and 5A). In light of these findings, we propose that the atherosclerotic microenvironment, which includes hypercholesterolemia and the induction of inflamma-
Fig. 5. Fortilin deficiency increases MΦ apoptosis and decreases the number of MΦ in atherosclerotic aortae. Red arrows show the MΦ population. A–F: flow cytometric analysis of atherosclerotic aortae from fortilin+/+ Ldlr−/− Apobec1−/− and fortilin−/− Ldlr−/− Apobec1−/− mice evaluating the total number of MΦ per milligram of tissue (A and B), the number of live [7-amino-actinomycin D (7-AAD) negative] MΦ (C and D), and the number of apoptotic (annexin V-positive) MΦ in the atherosclerotic aortae (E and F). Three aortae from each of the two genotypes were pooled, microdissected into small pieces, and subjected to enzymatic digestion and density gradient purification. Cells were stained with anti-CD11b-phycocerythrin (MΦ), anti-CD3e-allophycocyanin (T cells), and anti-CD19-allophycocyanin (B cells) antibodies as well as with peridinin chlorophyll protein-Cy 5.5 annexin V and 7-AAD. AU, arbitrary units. *P < 0.05.
tory cytokines such as M-CSF, induces the expression of fortilin in macrophages. This induction of fortilin protects macrophages from Bax-induced apoptosis and allows macrophages to propagate in the intima of the artery, eventually leading to accelerated atherosclerosis (Fig. 6).

How does fortilin protect macrophages against apoptosis? We recently reported that fortilin specifically binds tumor suppressor protein p53 and prevents it from transcriptionally activating Bax (8). The possibility that fortilin facilitates atherosclerosis by inhibiting p53 and Bax is supported by the fact that the lack of p53—more specifically, the lack of p53 in macrophage—causes accelerated atherosclerosis (17, 31, 42, 45). It is tempting to postulate that the disruption of the interaction between fortilin and p53 by small molecules could potentially reactivate p53, which would, in turn, protect the arteries against atherosclerotic changes.

In conclusion, the present work, for the first time, unequivocally shows the facilitative role of fortilin in atherosclerosis by inhibiting p53 and Bax.
and sheds new light on the mechanism of atherosclerosis by showing fortilin to be an important contributor of atherosclerosis. From the translational research standpoint, the findings of this study strongly suggest that fortilin is a viable molecular target for anti-atherosclerosis therapy, especially when a moderate reduction in fortilin expression (28–35%; Fig. 2, B–D) was sufficient to result in a significant reduction in atherosclerosis (Fig. 3). In other words, the development of agents that specifically inhibit fortilin could provide the basis for a novel, effective treatment for patients with this disease. Small molecules that disrupt the fortilin-p53 interaction could also have similar anti-atherosclerotic effects for the reason described above. Such inhibitors may facilitate the apoptosis of macrophages resident within the atherosclerotic lesion that overexpress fortilin. The ability to induce apoptosis on macrophages in the atherosclerotic intima would essentially equate to the elimination of atherosclerosis-promoting cells, which would otherwise proliferate, take up more lipids, and augment inflammation by secreting proinflammatory cytokines. Further investigation is now necessary to identify such agents and test their safety and efficacy in appropriate animal models of atherosclerosis.

GRANTS

This work was supported in part by National Heart, Lung, and Blood Institute Grants HL-68024 and HL-117247 (to K. Fujise), American Heart Association Established Investigator Award 0540054N (to K. Fujise), and American Heart Association Grant-In-Aid 7770000 (to K. Fujise).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: D.P. and K.F. conception and design of research; D.P., R.J.L., Y.C., M.E., B.-B.T., and K.F. performed experiments; D.P., R.J.L., Y.C., M.E., B.-B.T., and K.F. analyzed data; D.P., R.J.L., Y.C., B.-B.T., and K.F. interpreted results of experiments; D.P., R.J.L., Y.C., M.E., B.-B.T., and K.F. approved final version of manuscript; K.F. revised manuscript.

REFERENCES


2. Atkinson RD, Coenen KR, Plummer MR, Gruen ML, Hasty AH. No conflicts of interest, financial or otherwise, are declared by the author(s).


