ORIGINAL RESEARCH

Ectopic Fatty Acid–Binding Protein 4 Expression in the Vascular Endothelium is Involved in Neointima Formation After Vascular Injury

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***Background*—**Fatty acid-binding protein 4 (FABP4) is expressed in adipocytes, macrophages, and endothelial cells of capillaries but not arteries. FABP4 is secreted from adipocytes in association with lipolysis, and an elevated circulating FABP4 level is associated with obesity, insulin resistance, and atherosclerosis. However, little is known about the link between FABP4 and endovascular injury. We investigated the involvement of ectopic FABP4 expression in endothelial cells in neointima hyperplasia after vascular injury.

***Methods and Results*—**Femoral arteries of 8-week-old male mice were subjected to wire-induced vascular injury. After 4 weeks, immunofluorescence staining showed that FABP4 was ectopically expressed in endothelial cells of the hyperplastic neointima. Neointima formation determined by intima area and intima to media ratio was significantly decreased in FABP4-defficient mice compared with that in wild-type mice. Adenovirus-mediated overexpression of FABP4 in human coronary artery endothelial cells (HCAECs) in vitro increased inflammatory cytokines and decreased phosphorylation of nitric oxide synthase 3. Furthermore, FABP4 was secreted from HCAECs. Treatment of human coronary smooth muscle cells or HCAECs with the conditioned medium of *Fabp4*-overexpressed HCAECs or recombinant FABP4 significantly increased gene expression of inflammatory cytokines and proliferation- and adhesion-related molecules in cells, promoted cell proliferation and migration of human coronary smooth muscle cells, and decreased phosphorylation of nitric oxide synthase 3 in HCAECs, which were attenuated in the presence of an anti-FABP4 antibody.

***Conclusions*—**Ectopic expression and secretion of FABP4 in vascular endothelial cells contribute to neointima formation after vascular injury. Suppression of ectopic FABP4 in the vascular endothelium would be a novel strategy against post-angioplasty vascular restenosis. **(*J Am Heart Assoc*. 2017;6:e006377. DOI: 10.1161/JAHA.117.006377.)**

Key Words: endothelial cell • fatty acid–binding protein • neointimal hyperplasia • smooth muscle cell • vascular inflammation • vascular remodeling

Neointima hyperplasia in coronary artery lesions subjected to percutaneous coronary intervention is a pathologic characteristic of restenosis.[1] Endovascular injury causes impairment and regeneration of endothelial cells and activates vascular smooth muscle cells and smooth muscle cell–like cells differentiated from bone marrow–derived progenitor cells in the media, leading to neointima formation by cell migration into the intima and proliferation.[2,3] Drug-eluting stents have proven to be more effective than bare metal stents in reducing the incidence of restenosis after percutaneous coronary intervention, but coronary restenosis and stent thrombosis remain significant problems.[4]

Fatty acid–binding proteins are 14- to 15-kDa cytosolic proteins that can reversibly bind to saturated and unsaturated long-chain fatty acids with high affinity.[5–7] It has been proposed that fatty acid–binding proteins facilitate the transport of lipids to specific compartments in the cell. Fatty acid-binding protein 4 (FABP4), known as adipocyte fatty acid–binding protein, or aP2, is expressed in both adipocytes and macrophages and plays an important role in the development of insulin resistance and atherosclerosis.[8–10] We previously demonstrated that inhibition of FABP4 in cells would be a novel therapeutic strategy against insulin resistance, diabetes mellitus, and atherosclerosis.[11]

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Received April 13, 2017; accepted July 12, 2017.

Clinical Perspective

What Is New?

- Ectopic expression of fatty acid-binding protein 4 (FABP4) in vascular endothelial cells and accompanying inflammation were associated with neointima formation in the artery after wire-induced endovascular injury.
- FABP4 was secreted from vascular endothelial cells.
- Secreted FABP4 derived from ectopic FABP4 expression in endothelial cells promoted proliferative and migratory responses of vascular smooth muscle cells and vascular endothelial dysfunction, which were cancelled by the use of anti-FABP4 antibody.

What Are the Clinical Implications?

- Ectopic expression and secretion of FABP4 in the vascular endothelium are associated with neointima formation after vascular injury through endothelial dysfunction and inflammation in vascular endothelial cells and proliferation, migration, and inflammation in vascular smooth muscle cells.
- Inhibition of FABP4 by a small molecule, neutralization of FABP4 by the use of an antibody, or blocking unidentified receptors of FABP4 would be beneficial for the prevention of post-angioplasty vascular restenosis.

It has recently been reported that FABP4 is secreted from adipocytes in association with lipolysis via a nonclassical secretion pathway,[12,13] although there are no typical secretory signal peptides in the sequence of FABP4.[5] FABP4 has also been shown to be secreted from macrophages,[14] although the predominant contributors of circulating FABP4 are adipocytes rather than macrophages.[12] Previous studies demonstrated that circulating FABP4 acts as an adipokine, an adipocyte-derived bioactive molecule, for the development of insulin resistance and atherosclerosis.[12,14] Furthermore, elevation of the circulating FABP4 level is associated with obesity, insulin resistance, hypertension, cardiac dysfunction, dyslipidemia, and atherosclerosis.[15–21] Several drugs for dyslipidemia, diabetes mellitus, and hypertension have been reported to modulate FABP4 levels.[22–28]

Other than adipocytes and macrophages, FABP4 is also expressed in endothelial cells of capillaries and small veins, but not arteries, in several mouse and human tissues including the heart and kidney.[29,30] We previously demonstrated that ectopic FABP4 expression in endothelial cells of the glomerulus is associated with progression of proteinuria and renal dysfunction[31] and that urinary excretion of FABP4 would be a novel biomarker of glomerular damage.[32] Interestingly, it has been reported that FABP4 is markedly and ectopically upregulated in artery endothelial cells regenerating after endothelial balloon denudation in the pig coronary artery.[33] However, little is known about the link between FABP4 and endovascular injury. We therefore investigated the involvement of ectopic FABP4 expression in endothelial cells of neointima hyperplasia after endovascular injury by using a mouse model of wire-induced femoral artery injury, which mimics vascular remodeling following coronary angioplasty,[34] and by performing in vitro experiments using co-culture models and treatment with recombinant FABP4 and anti-FABP4 antibody in vascular endothelial cells and smooth muscle cells.

Methods

Biochemical Reagents and Animals

All experimental protocols were approved by the Animal Care Committee of Sapporo Medical University, and animal care and experimental procedures were performed in accordance with the Animal Care Committee of Sapporo Medical University. All biochemical reagents were purchased from Sigma-Aldrich unless indicated otherwise. Male C57BL/6J mice were obtained from Oriental Yeast. FABP4-deficient (*Fabp4*$^{-/-}$) and wild-type (*Fabp4*$^{+/+}$) mice were generated in the laboratory of Dr Gökhan S. Hotamisligil (Harvard T.H. Chan School of Public Health) and backcrossed for more than 8 generations into the C56BL/6J genetic background. Mice were kept on a 12-hour light cycle in a pathogen-free barrier facility and were placed on a regular chow diet ad libitum.

Wire Injury Model

Male mice aged 8 weeks were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg), and endoluminal injury of the left femoral artery was performed as previously described.[34,35] In brief, the left femoral artery was exposed and clamped upstream and downstream of the artery. From the branch of the femoral artery, a wire (diameter: 0.014 inches) was inserted into the artery and removed from the vessel followed by branch ligation.

Histological Analysis

Histological analysis was performed as previously described.[35,36] At 4 weeks after wire-induced injury, mice were anesthetized and fixed by perfusion with saline followed by 10% formalin through a cannula placed in the left ventricle. Both femoral arteries were excised from each mouse and embedded in paraffin. Neointima formation in the femoral arteries was evaluated at 10 locations at 100-μm intervals, with the most distal site located at the origin of the branch through which the wire had been inserted. The sections were stained by the Elastica-Van Gieson protocol. Images of arteries were captured with a digital color camera (DP21,

Table. Primers for Human and Mouse Genes in Quantitative Real-Time Polymerase Chain Reaction

Genes	Species	Accession No.	Forward Primer	Reverse Primer
18s	Human	M10098	5′-GTAACCCGTTGAACCCCATT-3′	5′-CCATCCAATCGGTAGTAGCG-3′
	Mouse	X00686	5′-AGTCCCTGCCCTTTGTACACA-3′	5′-CGATCCGAGGGCCTCACTA-3′
Fabp4	Human	NM_001442	5′-GCTTCCTTCTCACCTTGAAGAA-3′	5′-CCCACAGAATGTTGTAGAGTTC-3′
	Mouse	NM_024406	5′-AAGAAGTGGGAGTGGGCTTT-3′	5′-TCGACTTTCCATCCCACTTC-3′
Fabp5	Mouse	NM_010634	5′-CAAAACCGAGAGCACAGTGA-3′	5′-TTTGACCGCTCACTGAATTG-3′
Mcp1	Human	NM_002982	5′-CAGCCAGATGCAATCAATGCC-3′	5′-TGGAATCCTGAACCCACTTCT-3′
	Mouse	NM_011333	5′-CCACTCACCTGCTGCTACTCA-3′	5′-TGGTGATCCTCTTGTAGCTCTCC-3′
Il1b	Human	NM_000576	5′-CACGATGCACCTGTACGATCA-3′	5′-GTTGCTCCATATCCTGTCCCT-3′
	Mouse	NM_008361	5′-GAAATGCCACCTTTTGACAGTG-3′	5′-TGGATGCTCTCATCAGGACAG-3′
Il6	Human	NM_000600	5′-AAATTCGGTACATCCTCGACGG-3′	5′-GGAAGGTTCAGGTTGTTTTCTGC-3′
	Mouse	NM_031168	5′-ACAACCACGGCCTTCCCTACTT-3′	5′-CACGATTTCCCAGAGAACATGTG-3′
Tnfa	Human	NM_000594	5′-ATGAGCACTGAAAGCATGATCC-3′	5′-GAGGGCTGATTAGAGAGAGGTC-3′
	Mouse	NM_013693	5′-CCCTCACACTCAGATCATCTTCT-3′	5′-GCTACGACGTGGGCTACAG-3′
Icam1	Human	NM_000201	5′-ATGCCCAGACATCTGTGTCC-3′	5′-GGGGTCTCTATGCCCAACAA-3′
Sele	Human	NM_000450	5′-AGAGTGGAGCCTGGTCTTACA-3′	5′-CCTTTGCTGACAATAAGCACTGG-3′
Itga5	Human	NM_002205	5′-GGCTTCAACTTAGACGCGGAG-3′	5′-TGGCTGGTATTAGCCTTGGGT-3′
Itgb3	Human	NM_000212	5′-GTGACCTGAAGGAGAATCTGC-3′	5′-CCGGAGTGCAATCCTCTGG-3′
Pdgfra	Human	NM_006206.4	5′-AACCGTGTATAAGTCAGGGGA-3′	5′-GCATTGTGATGCCTTTGCCTT-3′
Pdgfrb	Human	NM_002609.3	5′-TCCAGCACCTTCGTTCTGAC-3′	5′-TATTCTCCCGTGTCTAGCCCA-3′

Olympus) mounted on a microscope (CX41, Olympus). Quantitative analysis for areas of the intima and media was performed using ImageJ software. All of the measurements were performed in a double-blind manner by 2 different researchers.

Immunofluorescence staining using rabbit anti-FABP4 (Abcam) and mouse anit-CD31 (Abcam) antibodies was performed as previously described.[35,36] Control experiments were performed by omitting the primary antibodies. Images were captured with a fluorescent microscope (BIOREVO BZ-9000 with a BZ-II analyzer, Keyence) or an LSM510META ConfoCor3 microscope (Carl Zeiss).

Quantitative Real-Time PCR

Total RNA was isolated using Trizol Reagent (Invitrogen). One microgram of total RNA was reverse-transcribed by using the High-Capacity cDNA Archive Kit (Applied Biosystems). Quantitative real-time PCR analysis was performed using SYBR Green in the real-time PCR system (Applied Biosystems). The thermal cycling program was 10 minutes at 95°C for enzyme activation and 40 cycles of denaturation for 15 seconds at 95°C, 30-second annealing at 58°C, and 30-second extension at 72°C. Primers used in the present study are listed in Table. To normalize expression data, 18s rRNA was used as an internal control gene.

Production and Purification of Recombinant FABP4 and Anti-FABP4 Antibody

Recombinant mouse FABP4 with a 6× His tag was produced in *Escherichia coli* using the pET21a vector (Novagen) and was purified with HisTrap HP (GE Healthcare) followed by endotoxin removal with a commercial system (Millipore). The rabbit polyclonal antibody against mouse FABP4 was produced using the recombinant full-length FABP4 protein, and the antibody was purified from the serum of the final bleed using the NAb Spin system (Pierce Biotechnology, Inc). Preimmune serum was purified similarly and used as a control.

Cell Culture

Human coronary artery endothelial cells (HCAECs) and human coronary artery smooth muscle cells (HCASMCs) were purchased from Lonza. HCAECs and HCASMCs were grown in Endothelial Cell Basal Medium-2 (EBM-2; Lonza) supplemented with EGM-2 MV SingleQuots (Lonza) and in Smooth Muscle Cell Basal Medium (Lonza) supplemented with SmGM-2 SingleQuots (Lonza), respectively, according to the manufacturer's instructions. After serum starvation for 24 hours, cells were stimulated with 50 ng/mL of vascular endothelial growth factor (VEGF) (R&D Systems) for 0.5 or 24 hours,

0.5 μmol/L insulin for 0.5 hours, 100 μmol/L H_2O_2 for 1 hour followed by 23-hour incubation in normal culture medium after washing, 0 to 200 nmol/L recombinant FABP4, or 10 μg/mL anti-FABP4 antibody in the medium supplemented with 0.5% BSA. The doses of reagents and incubation periods varied according to the experimental protocol. Each experiment was performed in at least triplicate.

Overexpression of FABP4 in HCAECs

HCAECs were infected with an adenovirus green fluorescent protein expressing vector-transfected human cDNA of FABP4 (Ad-FABP4) or an empty sequence (Ad-Control) as a control, which had been generated by Sirion Biotech. The cells were infected with the adenoviruses at a multiplicity of infection of 100 and a transduction enhancer (AdenoBoost, Sirion Biotech), and experiments were performed 2 days after infection. The conditioned medium (CM) was prepared by 24-hour incubation of HACECs transfected with Ad-FABP4 or Ad-Control in EBM-2 supplemented with 0.1% or 5% FBS or 0.5% BSA. Co-culture experiments were performed for HCASMCs and HCAECs treated with the CM of Ad-FABP4– and Ad-Control–transfected HCAECs or coincubated with Ad-FABP4– and Ad-Control–transfected HCAECs using insert transparent wells of a 1.0-μm pore size membrane (Falcon).

Assessment of FABP4 Secretion From Cultured HCAECs

After overnight serum depletion by 0.5% BSA in the medium, HCAECs were incubated with or without 10 μmol/L isopropanol in the medium supplemented with 0.5% BSA for 2 to 24 hours. The CM from the cells was filtered to obtain a 10- to 50-kDa fraction of proteins using Amicon Ultra 10K and 50K devices (Millipore). Total protein content of the cell lysate (CL) in a cell lysis buffer, containing 50 mmol/L Tris-HCl (pH 7.0), 2 mmol/L EGTA, 5 mmol/L EDTA, 30 mmol/L NaF, 10 mmol/L Na_3VO_4, 10 mmol/L $Na_4P_2O_7$, 40 mmol/L β-glycerophosphate, 0.5% NP-40, and 1% protease inhibitor cocktail, was assessed by a microplate protein assay based on Lowry's method (Bio-Rad). FABP4 protein in the CL and CM was determined by Western blotting and analyzed as previously described.[13,28]

FABP4 level in the CM was also measured using a commercially available enzyme-linked immunosorbent assay kit for FABP4 (Biovendor R&D). The intra-assay and interassay coefficients of variation in the kits were <5%. According to the manufacturer's protocol, no cross-reactivity of FABP4 with other fatty acid–binding protein types was observed. Secretion of FABP4 into the CM was normalized to total protein concentration of the CL.

Western Blotting

Total protein content of the samples was assessed by a microplate protein assay based on Lowry's method, and equal amounts of protein per sample and known molecular weight markers were subjected to SDS-PAGE. Proteins were electrophoretically transferred onto polyvinylidene fluoride membranes and incubated for 1 hour at room temperature with a blocking solution (3% BSA) in Tris-buffered saline buffer containing 0.1% Tween 20. The blocked membranes were incubated with primary antibodies for FABP4 (Abcam), GAPDH (Santa Cruz Biotechnology), nitric oxide synthase 3 (NOS3; BD Biosciences), phosphorylated NOS3 (BD Biosciences), and actin overnight at 4°C and washed 3 times with Tris-buffered saline buffer with 0.1% Tween 20. The membranes were incubated with a secondary antibody conjugated with horseradish peroxidase (GE Healthcare) for 1 hour at room temperature and washed. Immunodetection analyses were performed using a BM Chemiluminescence Blotting Substrate (POD) Kit (Roche Diagnostics). Densitometry was analyzed using ImageJ software.

Cell Proliferation and Migration Assays

An MTS assay was performed for assessing cell proliferation using the cell titer Aqueous One Solution Proliferation Assay (Promega). After overnight serum starvation by Smooth Muscle Cell Basal Medium with 0.1% FBS, HCASMCs were stimulated with the CM prepared by 24-hour incubation of Ad-FABP4– and Ad-Control–transfected HCAECs in EBM-2 supplemented with 5% FBS for 24 hours.

Cell proliferation was also analyzed by measuring DNA synthesis with a colorimetric bromodeoxyuridine (BrdU) enzyme-linked immunosorbent assay kit (Roche Diagnostics) according to the manufacturer's instructions. Briefly, 1×10^4 HCASMCs were seeded in a 96-well microplate and cultured with the CM prepared by 24-hour incubation of Ad-FABP4– and Ad-Control–transfected HCAECs in EBM-2 supplemented with 0.1% FBS or 0 to 200 nmol/L recombinant FABP4 supplemented with 0.1% FBS in the presence of 0 to 10 μg/mL anti-FABP4 antibody for 24 hours. The cells were then labeled with BrdU labeling reagent for 10 hours. After fixation, the cells were incubated with anti-BrdU antibody for 1.5 hours. After washing, 100 μL of a substrate (tetramethylbenzidine) was added to each well and the plates were incubated at room temperature for 0.5 hours. The absorbance at 450 nm was measured with an ELISA reader (Synergy H4, Biotek).

A scratch wound assay was performed for determining cell migration. After overnight serum starvation by Smooth Muscle Cell Basal Medium with 0.5% BSA, HCASMCs were scratched with a sterile pipette tip to produce a straight cell-free zone.

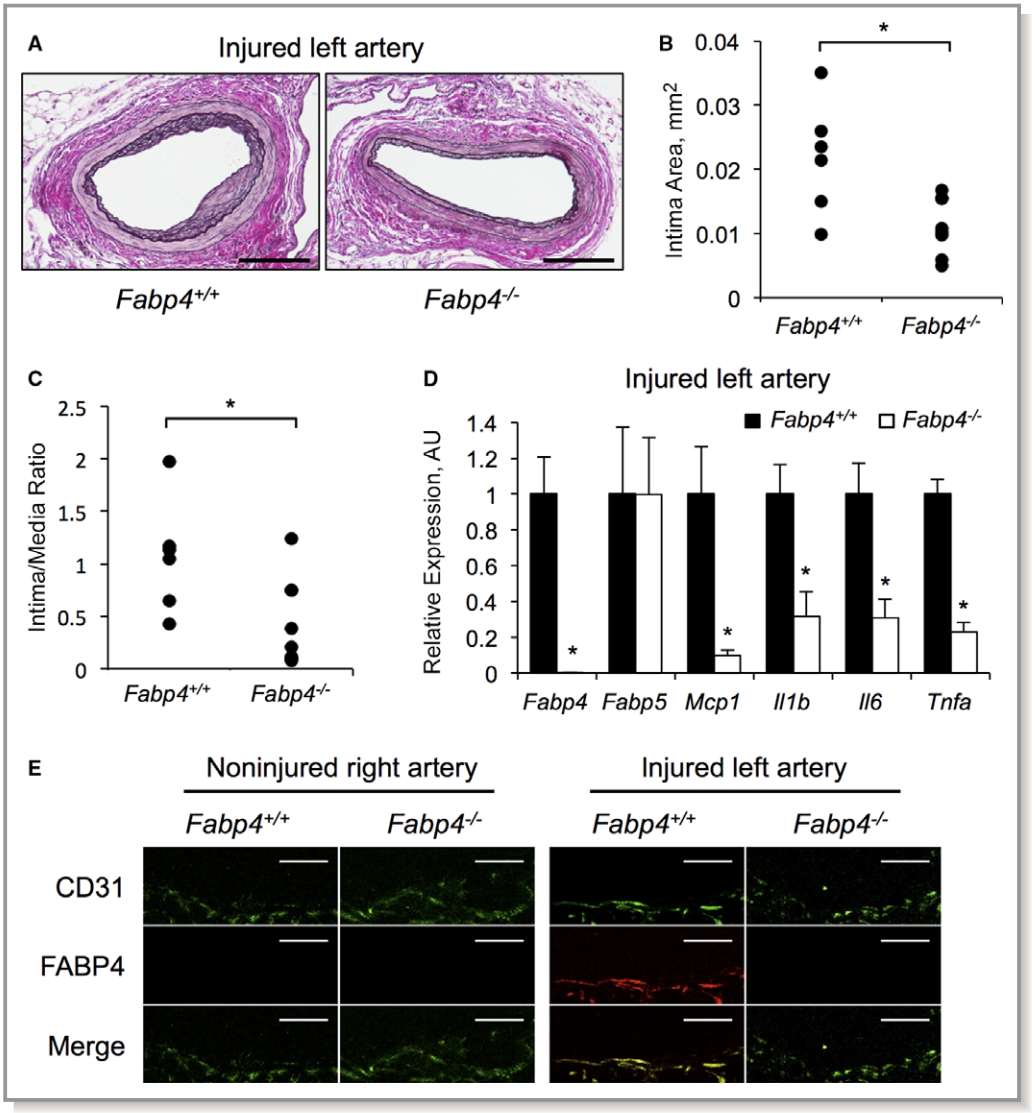

Figure 1. Neointima formation after vascular injury in fatty acid–binding protein 4 (FABP4)–deficient mice. A through C, Left femoral arteries of 8-week-old male wild-type (*Fabp4*$^{+/+}$) (n=6) and FABP4-deficient (*Fabp4*$^{-/-}$) (n=8) mice were subjected to wire-induced vascular injury. After 4 weeks, Elastica-Van Gieson staining was performed in the injured left femoral artery (A). Scale bars: 100 μm. The extent of neointima formation was evaluated as intima area (B) and intima to media ratio (C) in the wire-injured artery of *Fabp4*$^{+/+}$ and *Fabp4*$^{-/-}$ mice. *P<0.05. D, Gene expression levels of FABP4, fatty acid–binding protein 5 (FABP5), and inflammatory cytokines determined by quantitative real-time polymerase chain reaction in the wire-injured arteries of *Fabp4*$^{+/+}$ and *Fabp4*$^{-/-}$ mice (n=6 in each group). *P<0.05 vs *Fabp4*$^{+/+}$. E, Representative double immunofluorescence staining with CD31 (green) and FABP4 (red) in both the noninjured right and the injured left arteries. Scale bars: 20 μm. AU indicates arbitrary unit; IL, interleukin; Mcp1, monocyte chemotactic protein-1; Tnfa, tumor necrosis factor α.

The cells were stimulated with the CM prepared by 24-hour incubation of Ad-FABP4– and Ad-Control–transfected HCAECs in EBM-2 supplemented with 5% FBS or 100 nmol/L recombinant FABP4 supplemented with 0.5% BSA in the presence and absence of 10 μg/mL anti-FABP4 antibody for 15 hours. Pictures were taken at baseline and after stimulation, and migration distance was measured using ImageJ software as previously described.[35]

Statistical Analysis

Numeric variables are expressed as means±SEM. After confirming normal distribution of each variable, differences of means were analyzed using 1-way ANOVA with the Tukey-Kramer post hoc test for multiple groups and the Student *t* test for 2 groups. *P* values <0.05 were considered significant. All data were analyzed using JMP 9 for Macintosh (SAS Institute Inc).

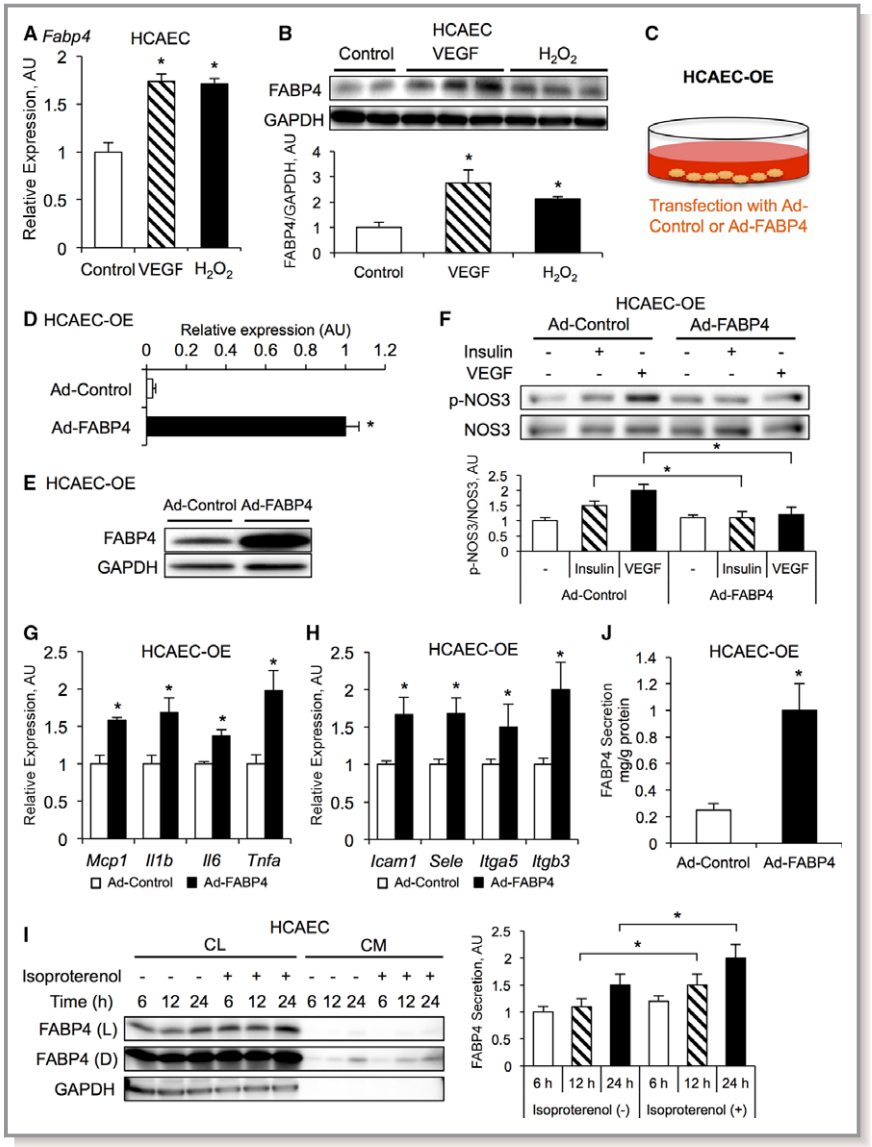

Figure 2. Overexpression and secretion of fatty acid–binding protein 4 (FABP4) in vascular endothelial cells. A and B, Gene (A) and protein (B) expression levels of FABP4 in human coronary artery endothelial cells (HCAECs) treated with 50 ng/mL of vascular endothelial growth factor (VEGF) for 24 hours or 100 μmol/L H_2O_2 for 1 hour followed by 23-hour incubation in normal culture media after washing were determined by quantitative real-time polymerase chain reaction (n=3 in each group) and Western blot analysis, respectively. *P<0.05 vs control. C, Experimental design of adenovirus-mediated overexpression in HCAECs using adenovirus vector of FABP4 (Ad-FABP4) or empty sequence (Ad-Control) (HCAEC-OE). D, Gene expression of FABP4 in HCAEC-OE (n=6 in each group). *P<0.05 vs Ad-Control. E, Representative Western blot analysis of FABP4 in HCAEC-OE. F, Western blot analysis of nitric oxide synthase (NOS3) and phosphorylated NOS3 (pS1177) in HCAEC-OE treated with 0.5 μmol/L insulin or 50 ng/mL VEGF for 0.5 hours (n=3 in each group). *P<0.05. G and H, Gene expression of inflammatory cytokines (G) and adhesion-related molecules (H) in HCAEC-OE (n=6 in each group). *P<0.05 vs Ad-Control. I, Western blot analysis of FABP4 (exposure: light, L; dark, D) and GAPDH was performed using the cell lysate (CL) and conditioned medium (CM) of HCAECs supplemented with 0.5% BSA in the absence and presence of 10 μmol/L isoproterenol for 6 to 24 hours (n=3 in each group). *P<0.05. J, Secretion of FABP4 for 24 hours in HCAEC-OE measured using an enzyme-linked immunosorbent assay kit (n=3 in each group). Values were normalized to total protein concentration of the cell lysate. *P<0.05 vs Ad-Control. AU indicates arbitrary unit; Icam1, including intracellular adhesion; IL, interleukin; Itga5, integrin a 5; Itgb3, integrin b 3; Mcp1, monocyte chemotactic protein-1; Sele, selectin E; Tnfa, tumor necrosis factor α.

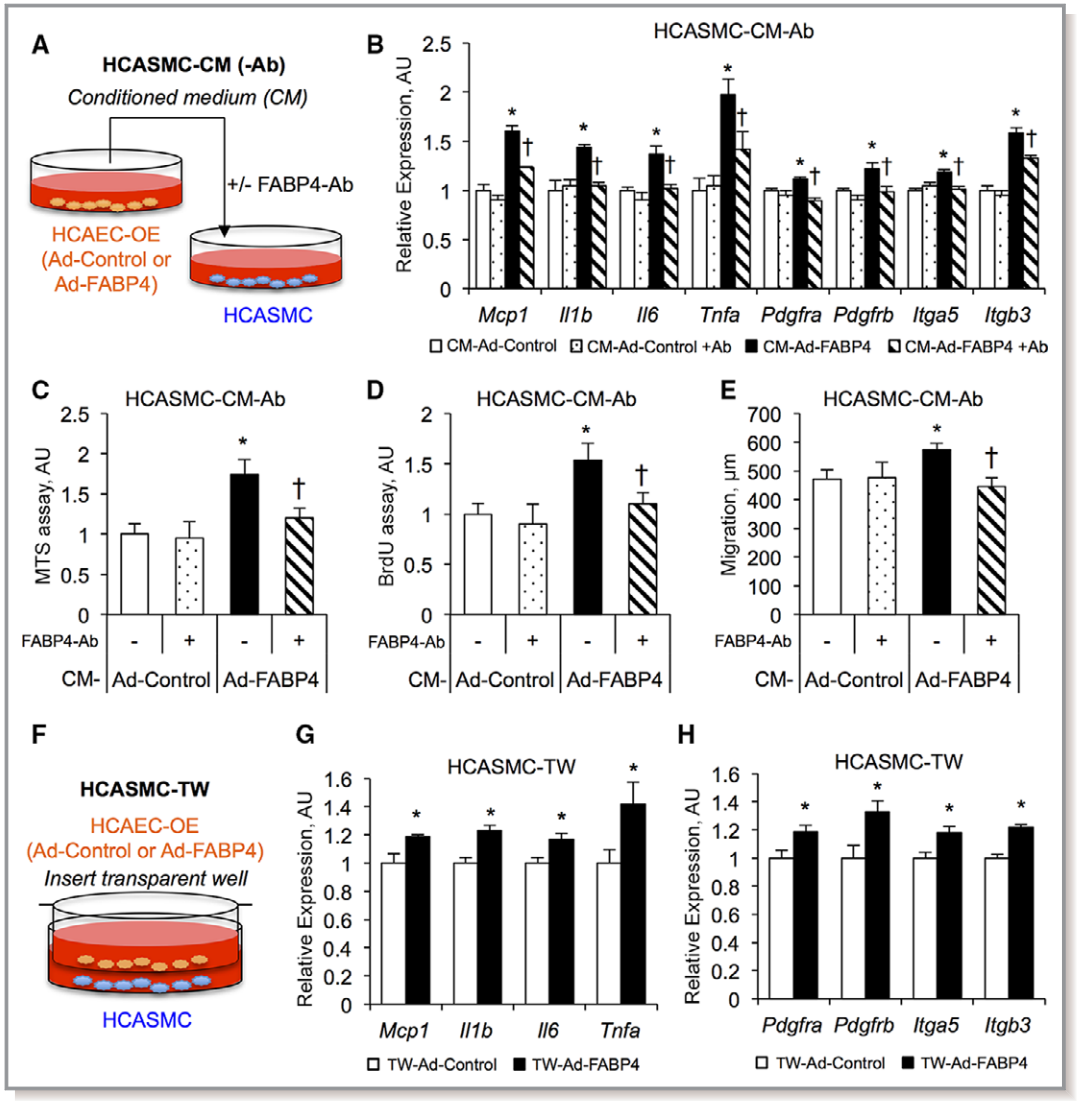

Figure 3. Effects of the conditioned medium of *Fabp4*-overexpressed endothelial cells in vascular smooth muscle cells. A, Experimental design of human coronary artery smooth muscle cells (HCASMCs) treated with the conditioned medium (CM) prepared by 24-hour incubation of adenovirus vector of fatty acid–binding protein 4 (Ad-FABP4)– and empty sequence (Ad-Control)–transfected human coronary artery endothelial cells (HCAECs) in the absence and presence of 10 μg/mL anti-FABP4 antibody (FABP4-Ab) for 24 hours (HCASMC-CM-Ab). B, Gene expression of inflammatory cytokines and proliferation- and adhesion-related molecules determined by quantitative real-time polymerase chain reaction (PCR) in HCASMC-CM-Ab (n=6 in each group). **P*<0.05 vs CM of Ad-Control–transfected HCAECs (CM-Ad-Control). †*P*<0.05 vs CM of Ad-FABP4–transfected HCAECs (CM-Ad-FABP4). C and D, Proliferation of HCASMC-CM supplemented with 5% FBS in the absence and presence of 10 μg/mL FABP4-Ab for 24 hours assessed by MTS (C) and bromodeoxyuridine (BrdU; D) assays (n=6 in each group). **P*<0.05 vs CM-Ad-Control. †*P*<0.05 vs CM-Ad-FABP4. E, Migration of HCASMC-CM supplemented with 0.5% BSA in the absence and presence of 10 μg/mL FABP4-Ab for 15 hours assessed by scratch wound assay (n=6 in each group). **P*<0.05 vs CM-Ad-Control. †*P*<0.05 vs CM-Ad-FABP4. F, Experimental design of HCASMCs coincubated with Ad-FABP4– and Ad-Control–transfected HCAECs using insert transparent wells for 24 hours (HCASMC-TW). G and H, Gene expression of inflammatory cytokines (G) and proliferation- and adhesion-related molecules (H) determined by quantitative real-time PCR in HCASMC-TW (n=6 in each group). **P*<0.05 vs coincubation using transparent wells with Ad-Control–overexpressed HCAECs (TW-Ad-Control). AU indicates arbitrary unit; IL, interleukin; Itga5, integrin a 5; Itgb3, integrin b 3; Mcp 1, monocyte chemotactic protein-1; Pdgfra, platelet-derived growth factor receptor α; Pdgfrb, platelet-derived growth factor receptor β; Tnfa, tumor necrosis factor α.

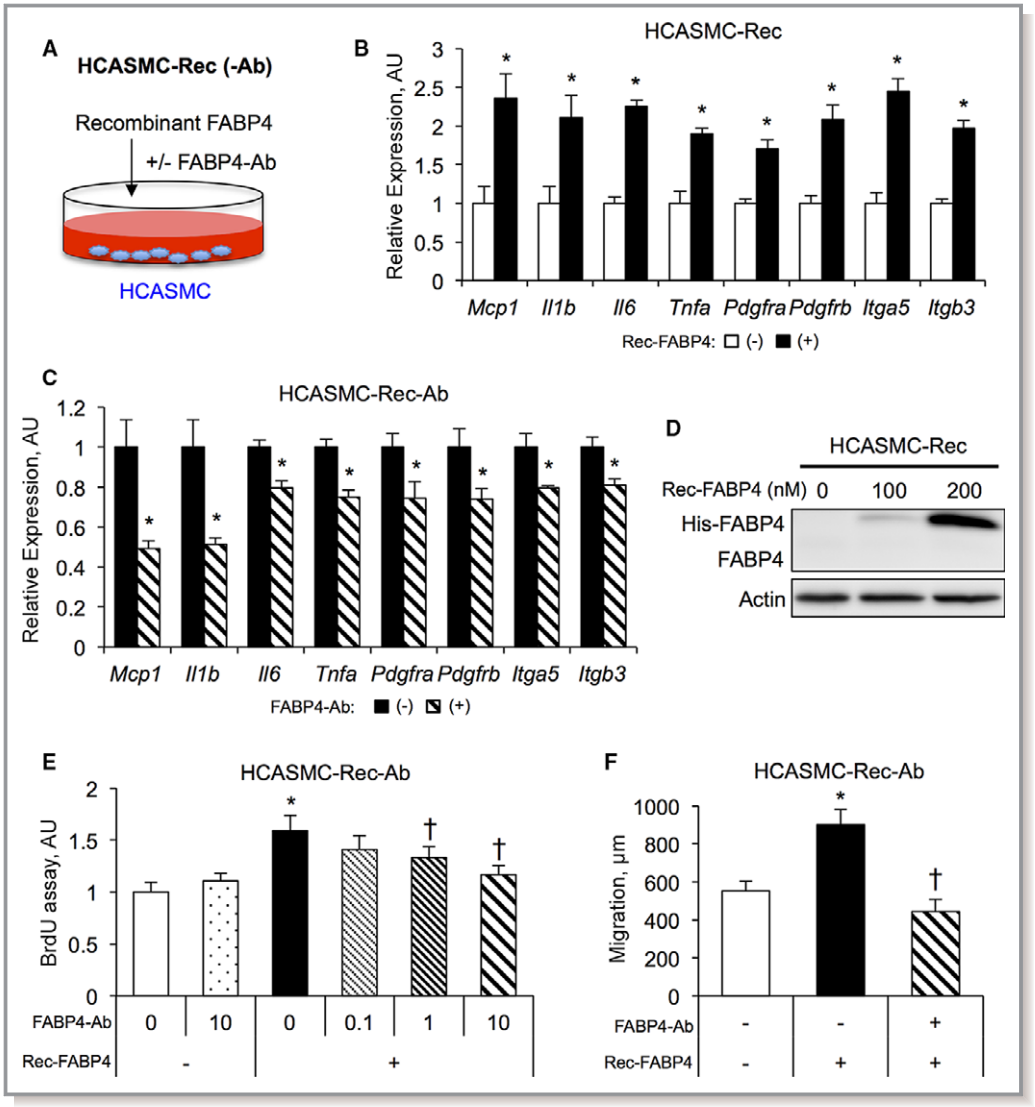

Figure 4. Effects of exogenous fatty acid–binding protein 4 (FABP4) treatment in vascular smooth muscle cells. A, Experimental design of human coronary artery smooth muscle cells (HCASMCs) treated with 0 to 200 nmol/L recombinant FABP4 (HCASMC-Rec) in the absence and presence of 0.1 to 10 μg/mL anti-FABP4 antibody (FABP4-Ab) for 24 hours (HCASMC-Rec-Ab). B, Gene expression of inflammatory cytokines and proliferation- and adhesion-related molecules determined by quantitative real-time polymerase chain reaction (PCR) in HCASMCs treated with 200 nmol/L recombinant FABP4 (Rec-FABP4) for 24 hours (n=4 in each group). **P*<0.05 vs Rec-FABP4 (−). C, Gene expression of inflammatory cytokines and proliferation- and adhesion-related molecules determined by quantitative real-time PCR in HCASMCs treated with 200 nmol/L of Rec-FABP4 in the absence and presence of 10 μg/mL FABP4-Ab for 24 hours (n=4 in each group). **P*<0.05 vs FABP4-Ab (−). D, Representative Western blot analysis of endogenous FABP4, His-tagged FABP4 (His-FABP4), and GAPDH in HCASMCs treated with 0 to 200 nmol/L His-tagged Rec-FABP4 for 24 hours. E, Proliferation of HCASMCs supplemented with 0.1% FBS in the absence and presence of 200 nmol/L Rec-FABP4 and 0 to 10 μg/mL of FABP4-Ab for 24 hours (HCASMC-Rec-Ab) assessed by bromodeoxyuridine (BrdU) assay (n=6 in each group). **P*<0.05 vs Rec-FABP4 (−) and FABP4-Ab (0 μg/mL). †*P*<0.05 vs Rec-FABP4 (+) and FABP4-Ab (0 μg/mL). F, Migration of HCASMCs supplemented with 0.5% BSA in the absence and presence of 200 nmol/L Rec-FABP4 and 10 μg/mL FABP4-Ab for 15 hours assessed by scratch wound assay (n=6 in each group). **P*<0.05 vs Rec-FABP4 (−) and FABP4-Ab (−). †*P*<0.05 vs Rec-FABP4 (+) and FABP4-Ab (−). AU indicates arbitrary unit; IL, interleukin; Itga5, integrin a 5; Itgb3, integrin b 3; Mcp1, monocyte chemotactic protein-1; Pdgfra, platelet-derived growth factor receptor α; Pdgfrb, platelet-derived growth factor receptor β; Tnfa, tumor necrosis factor α.

Results

Involvement of Ectopic FABP4 Expression in Endothelial Cells in Neointima Formation

The left femoral arteries of 8-week-old male *Fabp4*$^{+/+}$ and *Fabp4*$^{-/-}$ mice were subjected to wire-induced vascular injury to investigate the association between vascular remodeling and ectopic expression of FABP4 in vivo. Four weeks later, wire injury-mediated neointima hyperplasia was induced, and the thickness of the neointima determined by intima area and intima to media ratio was significantly smaller in *FABP4*$^{-/-}$ mice than in *Fabp4*$^{+/+}$ mice (Figure 1A through 1C). Gene expression levels of fatty acid–binding protein 5 were comparable in the injured arteries of *Fabp4*$^{+/+}$ and *Fabp4*$^{-/-}$ mice, but *Fabp4*$^{-/-}$ mice had significantly lower gene expression levels of inflammatory cytokines in the injured arteries, including MCP-1 (monocyte chemotactic protein-1), interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α), than did *Fabp4*$^{+/+}$ mice (Figure 1D). Immunofluorescence staining showed that FABP4 was absent in noninjured arteries of *Fabp4*$^{+/+}$ and *Fabp4*$^{-/-}$ mice but was present in cells stained by CD31, a maker of endothelial cells, of the hyperplastic neointima in the injured arteries of *Fabp4*$^{+/+}$ mice (Figure 1E).

Effects of FABP4 Overexpression in Vascular Endothelial Cells

Gene (Figure 2A) and protein (Figure 2B) expressions of FABP4 were induced by treatment of HCAECs with VEGF and H_2O_2 as previously reported in endothelial cells.[29,37] Adenovirus-mediated *Fabp4* overexpression in HCAECs (Figure 2C) resulted in efficient gene and protein induction of FABP4 (Figure 2D and 2E) and decreased phosphorylation of NOS3 by stimulation with insulin or VEGF (Figure 2F). Overexpression of *Fabp4* in HCAECs significantly increased gene expression levels of inflammatory cytokines, including *Mcp1*, *Il1b*, *Il6*, and *Tnfa* (Figure 2G), and adhesion-related molecules, including intracellular adhesion molecule–1 (ICAM-1), selectin E (SELE), integrin α 5 (ITGA5), and integrin β 3 (ITGB3) (Figure 2H).

Secretion of FABP4 From Vascular Endothelial Cells

Results from Western blot analysis showed that FABP4 was present in both the CL and CM of HCAECs and that GAPDH, a nonsecretory protein, was not present in the CM (Figure 2I), indicating that FABP4 in the CM was a result of its secretion from HCAECs, not a result of its leakage via injured cell membranes. FABP4 was secreted from HCAECs in a time-dependent manner (Figure 2I), and the secretion of FABP4 was slightly, but significantly, enhanced in the presence of isoproterenol for 12 and 24 hours, which is known to increase secretion of FABP4 from adipocytes.[12,13] Secretion of FABP4 in the CM of Ad-FABP4–transfected HCAECs was significantly greater than that in the CM of Ad-Control–transfected HCAECs (Figure 2J).

Effects of HCASMCs Coincubated With *Fabp4*-Overexpressed HCAEC

Incubation of HCASMCs with the CM of *Fabp4*-overexpressed HCAECs (Figure 3A) significantly increased gene expression levels of inflammatory cytokines, including *Mcp1*, *Il1b*, *Il6*, and *Tnfa*; proliferation-related molecules, including platelet-derived growth factor receptor α and β; and adhesion-related molecules, including *Itga5* and *Itgb3*, which were attenuated in the presence of anti-FABP4 antibody (Figure 3B). MTS and BrdU assays showed that incubation of HCASMCs with the CM of *Fabp4*-overexpressed HCAECs significantly increased cell proliferation, which was cancelled by coincubation with anti-FABP4 antibody (Figure 3C and 3D). The scratch wound–healing assay showed that HCASMCs incubated with the CM of *Fabp4*-overexpressed HCAECs migrated faster than did those incubated with the CM of control cells, which was cancelled by coincubation with anti-FABP4 antibody (Figure 3E).

In HCASMCs coincubated with *Fabp4*-overexpressed HCAECs using insert transparent wells (Figure 3F), similar results were obtained for gene expression levels of inflammatory cytokines (Figure 3G) and proliferation- and adhesion-related molecules (Figure 3H).

Treatment of HCASMCs With Recombinant FABP4 and Anti-FABP4 Antibody

Treatment of HCASMCs with recombinant FABP4 (Figure 4A; HCASMC-Rec) significantly increased gene expression levels of inflammatory cytokines and proliferation- and adhesion-related molecules (Figure 4B), which were attenuated in the presence of anti-FABP4 antibody (Figure 4C). Western blot analysis showed that recombinant FABP4 with a 6× His tag was partially internalized into HCASMCs, although endogenous expression of FABP4 was absent in HCASMCs (Figure 4D), indicating possible effects of exogenous FABP4 on HACSMCs in both extracellular and intracellular manners. A BrdU assay showed that treatment of HCASMCs with recombinant FABP4 significantly increased cell proliferation, which was attenuated by coincubation with anti-FABP4 antibody in a dose-dependent manner (Figure 4E). The scratch wound–healing assay showed that FABP4-treated HCASMCs migrated faster than did untreated cells, which was cancelled by coincubation with anti-FABP4 antibody (Figure 4F).

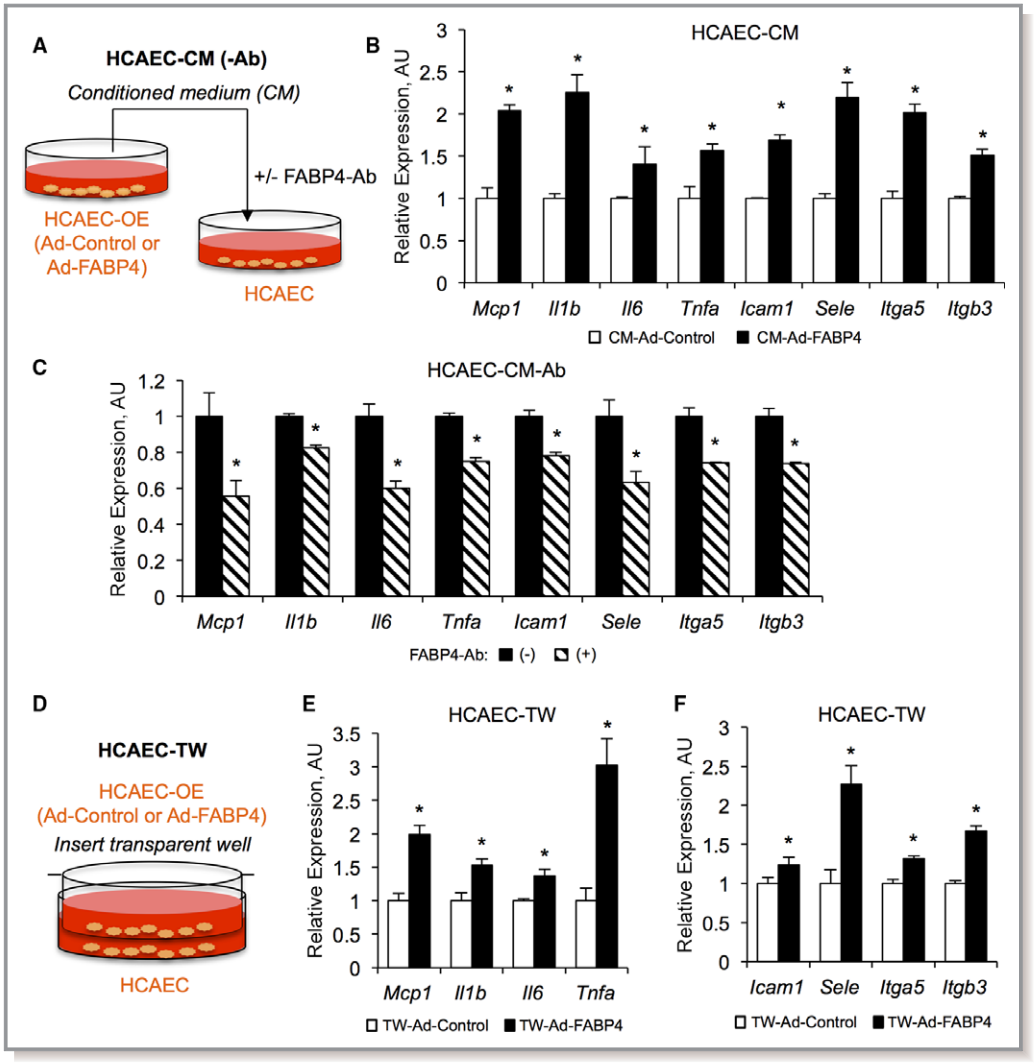

Figure 5. Effects of the conditioned medium of *Fabp4*-overexpressed endothelial cells in vascular endothelial cells. A, Experimental design of human coronary artery endothelial cells (HCAECs) treated with the conditioned medium (CM) prepared by 24-hour incubation of adenovirus vector of fatty acid–binding protein 4 (Ad-FABP4)– and empty sequence (Ad-Control)–transfected HCAECs (HCAEC-CM) in the absence and presence of 10 μg/mL anti-FABP4 antibody (FABP4-Ab) for 24 hours (HCAEC-CM-Ab). B, Gene expression of inflammatory cytokines and adhesion-related molecules determined by quantitative real-time polymerase chain reaction (PCR) in HCAEC-CM (n=6 in each group). **P*<0.05 vs CM of Ad-Control–overexpressed HCAECs (CM-Ad-Control). C, Gene expression of inflammatory cytokines and adhesion-related molecules determined by quantitative real-time PCR in HCAEC-CM-Ab (n=4 in each group). **P*<0.05 vs FABP4-Ab (−). D, Experimental design of HCAECs coincubated with Ad-FABP4– and Ad-Control–transfected HCAECs using insert transparent wells for 24 hours (HCAEC-TW). E and F, Gene expression of inflammatory cytokines (E) and adhesion-related molecules (F) determined by quantitative real-time PCR in HCAEC-TW (n=6 in each group). **P*<0.05 vs coincubation using transparent wells with Ad-Control–overexpressed HCAECs (TW-Ad-Control). AU indicates arbitrary unit; Icam1, including intracellular adhesion; IL, interleukin; Itga5, integrin a 5; Itgb3, integrin b 3; Mcp1, monocyte chemotactic protein-1; Sele, selectin E; Tnfa, tumor necrosis factor α.

Effects of HCAECs Coincubated With Fabp4-Overexpressed HCAECs

Incubation of HCAECs with the CM of *Fabp4*-overexpressed HCAECs (Figure 5A) significantly increased gene expression levels of inflammatory cytokines, including *Mcp1*, *Il1b*, *Il6*, and *Tnfa*, and adhesion-related molecules, including *Icam1*, *Sele* *Itga5*, and *Itgb3* (Figure 5B), which were attenuated in the presence of anti-FABP4 antibody (Figure 5C).

Similarly, in HCAECs coincubated with *Fabp4*-overexpressed HCAECs using insert transparent wells (Figure 5D), gene expression levels of inflammatory cytokines

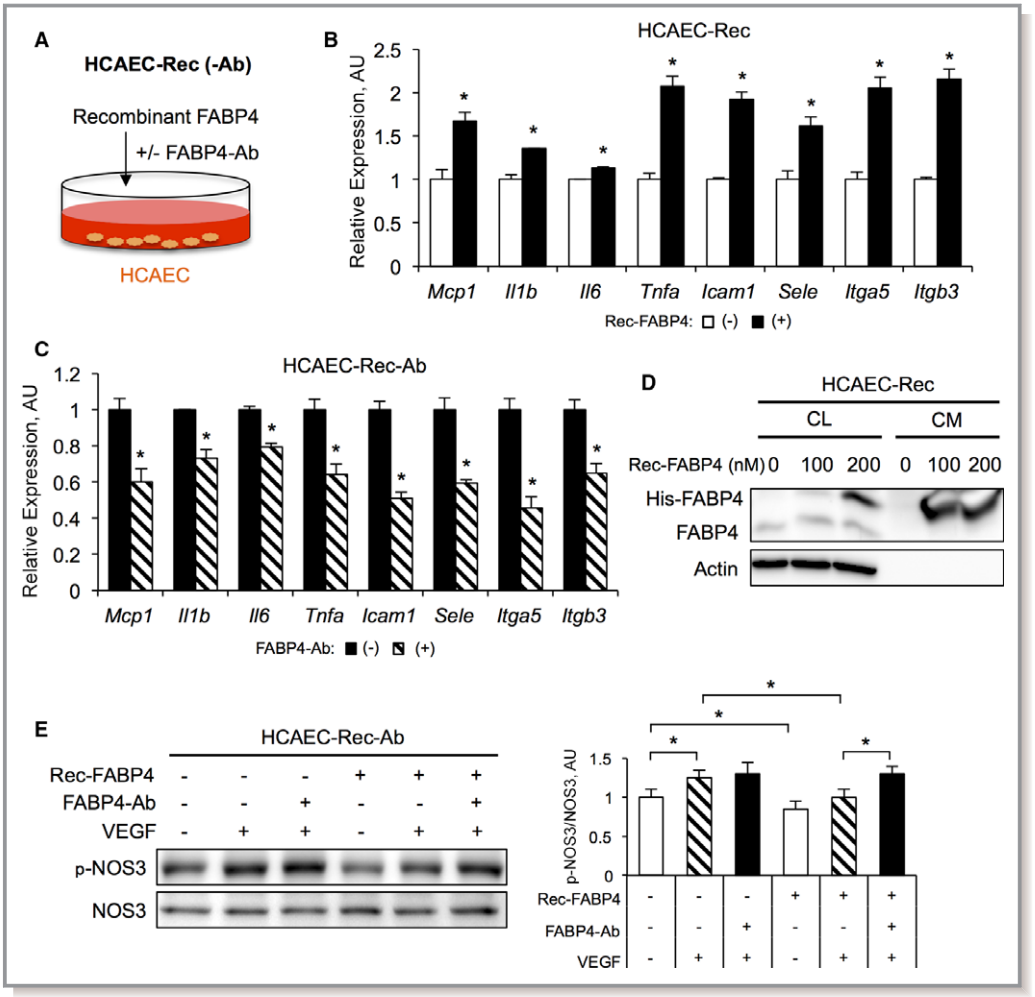

Figure 6. Effects of exogenous fatty acid–binding protein 4 (FABP4) treatment in vascular endothelial cells. A, Experimental design of human coronary artery endothelial cells (HCAECs) treated with 200 nmol/L recombinant FABP4 (HCAEC-Rec) in the absence and presence of 10 μg/mL anti-FABP4 antibody (FABP4-Ab) for 24 hours (HCAEC-Rec-Ab). B, Gene expression of inflammatory cytokines and adhesion-related molecules determined by quantitative real-time polymerase chain reaction (PCR) in HCAEC-Rec (n=3 in each group). **P*<0.05 vs Rec-FABP4 (−). C, Gene expression of inflammatory cytokines and adhesion-related molecules determined by quantitative real-time PCR in HCAEC-Rec-Ab (n=3 in each group). **P*<0.05 vs FABP4-Ab (−). D, Representative Western blot analysis of endogenous FABP4, His-tagged FABP4 (His-FABP4), and GAPDH was performed using the cell lysate (CL) and conditioned medium (CM) of HCAECs treated with 0 to 200 nmol/L His-tagged Rec-FABP4 for 6 hours. E, Western blot analysis of nitric oxide synthase 3 (NOS3) phosphorylated NOS3 (pS1177) in HCAEC-Rec-Ab followed by stimulation with 50 ng/mL vascular endothelial growth factor (VEGF) for 0.5 hours (n=3 in each group). **P*<0.05. AU indicates arbitrary unit; Icam1, including intracellular adhesion; IL, interleukin; Itga5, integrin a 5; Itgb3, integrin b 3; Mcp1, monocyte chemotactic protein-1; Sele, selectin E; Tnfa, tumor necrosis factor α.

(Figure 5E) and adhesion-related molecules (Figure 5F) were increased.

Treatment of HCAECs With Recombinant FABP4 and Anti-FABP4 Antibody

Treatment of HCAECs with recombinant FABP4 (Figure 6A) increased gene expression levels of inflammatory cytokines, including *Mcp1*, *Il1b*, *Il6*, and *Tnfa*, and adhesion-related molecules, including *Icam1*, *Sele*, *Itga5*, and *Itgb3* (Figure 6B), which were attenuated by coincubation with anti-FABP4 antibody (Figure 6C). Similarly in HCASMCs, findings from Western blot analysis showed that recombinant FABP4 with a 6× His tag was partially internalized into HCAECs (Figure 6D), indicating possible effects of exogenous FABP4 on HCAECs in both extracellular and intracellular manners. Treatment of HCAECs with recombinant FABP4 decreased

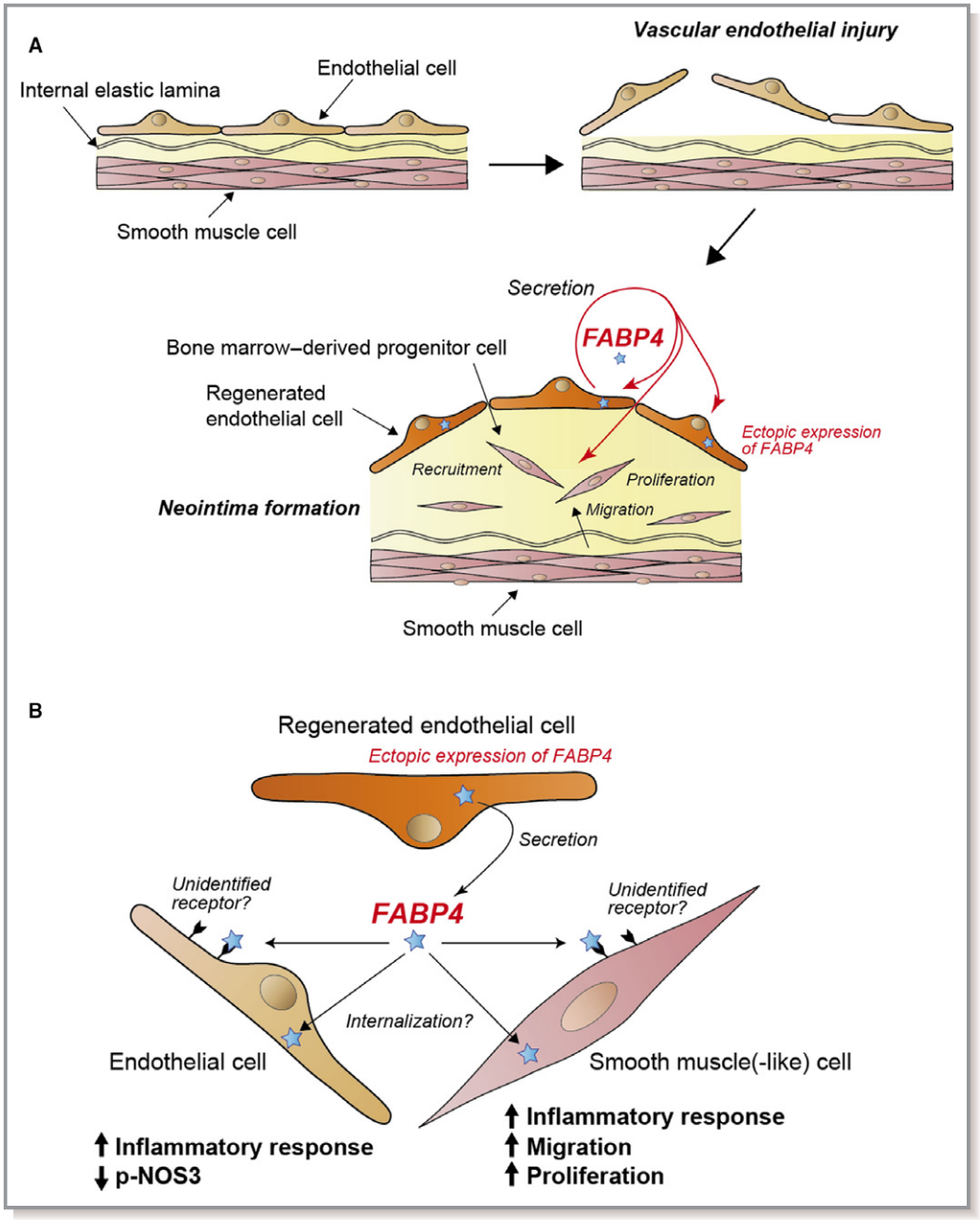

Figure 7. Putative mechanism of ectopic fatty acid–binding protein 4 (FABP4) expression in the endothelium underlying the development of neointima formation after endovascular injury. A, FABP4 is locally secreted by ectopic expression of FABP4 in vascular regenerated endothelial cells of the hyperplastic neointima after vascular injury. The secreted FABP4 acts as a biological molecule in nearby cells, including vascular endothelial cells, vascular smooth muscle cells, and smooth muscle–like cells differentiated from bone marrow–derived progenitor cells, in autocrine and paracrine manners. B, Local production of FABP4 derived from regenerated endothelial cells after endovascular injury may act in cells through unidentified receptors and/or internalization into the cells, leading to the development of neointima formation by cooperating with accelerating vascular inflammation, proliferation and migration of smooth muscle cells, and impaired endothelial function. p-NOS3 indicates phosphorylated nitric oxide synthase 3.

basal and VEGF-stimulated phosphorylation of NOS3, and coincubation of recombinant FABP4 with anti-FABP4 antibody restored VEGF-stimulated phosphorylation of NOS3 (Figure 6E).

Discussion

We demonstrated for the first time that ectopic expression of FABP4 in vascular endothelial cells and accompanying

inflammation are associated with neointima formation in the artery after wire-induced endovascular injury. FABP4 was ectopically induced in endothelial cells of the artery after endovascular injury as previously reported,[33] and deletion of FABP4 significantly decreased neointima hyperplasia after wire-induced vascular injury in vivo. In contrast, overexpression of *Fabp4* in vascular endothelial cells increased the expression of inflammatory and adhesion-related genes and impaired endothelial function. Furthermore, FABP4 was secreted from endothelial cells. Proliferative and migratory responses of HCASMCs and endothelial dysfunction of HCAECs were enhanced by treatment with the CM of *Fabp4*-overexpressed endothelial cells or exogenous FABP4, which were cancelled by the use of anti-FABP4 antibody. Thus, the present findings collectively suggest that secreted FABP4 derived from ectopic expression of FABP4 in endothelial cells after vascular injury contributes to the progression of neoinitima formation in paracrine and autocrine manners, resulting in vascular restenosis. The putative mechanism of secreted FABP4 derived from ectopic FABP4 expression in endothelial cells underlying the development of neointima formation is shown in Figure 7.

The pathology of neointima formation is traditionally thought to be migration and proliferation in the media, which are affected by various elements derived from around immune and vascular cells, such as growth factors and inflammatory cytokines.[2] It has also been shown that smooth muscle cell–like cells differentiated from bone marrow–derived progenitor cells play important roles in neointima formation.[3] Transcriptome and metabolome analyses showed that exogenous FABP4 affects transcriptional and metabolic regulation in adipose-derived stem cells near adipocytes.[38] Furthermore, previous studies using in vitro and in vivo experiments showed that circulating FABP4 acts as an adipokine, an adipocyte-derived bioactive molecule, for the development of insulin resistance through increased hepatic glucose production[12] and for the development of atherosclerosis through inhibition of endothelial NOS activity in endothelial cells,[14,39] proliferation and migration of vascular smooth muscle cells,[14,40] and induction of inflammatory responses in macrophages, vascular smooth muscle cells, and vascular endothelial cells.[14] Similarly, we demonstrated in the present study that treatment of vascular smooth muscle cells and vascular endothelial cells with recombinant FABP4 induced vascular inflammation, proliferative and migratory responses, and endothelial dysfunction. Recent studies have also demonstrated that neutralization of secreted FABP4 with an antibody to FABP4 could be a feasible approach for treatment of insulin resistance and type 2 diabetes mellitus.[12,41] In the present study, we revealed inhibitory effects of FABP4 neutralization using an antibody to FABP4 on proliferation, migration, and inflammatory response in vascular smooth muscle cells and on inflammatory response and endothelial dysfunction in vascular endothelial cells in vitro. Circulating FABP4 derived from ectopic expression of FABP4 in the endothelium may directly affect neointima formation after endovascular injury.

FABP4 is secreted from adipocytes under regulation by the catecholamine-induced lipolytic signal pathway, although FABP4 lacks an N-terminal secretory signal sequence.[7,12,13] The present study showed that secretion of FABP4 from endothelial cells was slightly, but significantly, enhanced by stimulation of isoproterenol, similar to that in the case of adipocytes. It was previously shown that treatment with several inflammatory stimuli, including VEGF, H_2O_2 and cytokines, and cellular senescence induce expression of FABP4 in endothelial cells,[29,37] although the expression level of FABP4 in endothelial cells is lower than that in adipocytes.[7,30] It is possible that the level of FABP4 secreted from endothelial cells is sufficient for causing significant effects on nearby vascular endothelial cells and smooth muscle cells, leading to an inflammatory response, proliferation, and migration from the media into the intima. The circulating level of FABP4 is ≈20 ng/mL (1 nmol/L) in humans.[19] Since autocrine and paracrine actions of the secreted FABP4 derived from regenerated endothelial cells were focused on in the present study, the concentration of recombinant FABP4 used (≈200 nmol/L) seems to be reasonable and physiological in the local area.

Evidence indicating that FABP4 acts as a biological molecule is accumulating,[12,14,39–43] and serum FABP4 level has been reported to predict long-term cardiovascular events.[44–46] However, the receptor for FABP4 remains unknown. The present study showed that extracellular FABP4 is partially internalized into the cell, but it is unclear whether extracellular FABP4 acts by an intracellular signaling mechanism. A further understanding of the mechanism of FABP4 action may enable the development of new therapeutic strategies for cardiovascular and metabolic diseases as well as endovascular injury, such as neutralization of FABP4 and/or blockade of the FABP4 receptor, if any.

Conclusions

Ectopic expression and secretion of FABP4 in the vascular endothelium are associated with neointima formation after vascular injury through endothelial dysfunction and inflammation in vascular endothelial cells and proliferation, migration, and inflammation in vascular smooth muscle cells. A further understanding of ectopic expression of FABP4 from endothelial cells as well as adipocytes and macrophages may enable the development of new therapeutic strategies for cardiovascular and metabolic diseases. Inhibition of FABP4 by a small molecule, neutralization of FABP4 by the use of an

antibody, or blocking unidentified receptors of FABP4 would be beneficial for the prevention of post-angioplasty vascular restenosis.

Sources of Funding

Furuhashi has been supported by grants from JSPS KAKENHI, MEXT Translational Research Network Program, Uehara Memorial Foundation, SENSHIN Medical Research Foundation, Japan Diabetes Foundation, Takeda Medical Research Foundation, Ono Medical Research Foundation, Takeda Science Foundation, Akiyama Life Science Foundation, Yamaguchi Endocrine Research Foundation, Naito Foundation Natural Science Scholarship, Suhara Memorial Foundation, Kondou Kinen Medical Foundation, and Terumo Foundation for Life Science and Arts.

Disclosures

None.

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