EXPERIMENTAL STUDY

FABP5 Is a Sensitive Marker for Lipid-Rich Macrophages in the Luminal Side of Atherosclerotic Lesions

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Summary

Lipid-rich macrophages in atherosclerotic lesions are thought to be derived from myeloid and vascular smooth muscle cells. A series of studies with genetic and pharmacological inhibition of fatty acid binding protein 4 (FABP4) and FABP5 and bone marrow transplant experiments with FABP4/5 deficient cells in mice have demonstrated that these play an important role in the development of atherosclerosis. However, it is still uncertain about the differential cell-type specificity and distribution between FABP4 and FABP5-expressing cells in early- and late-stage atherosclerotic lesions. In this study, we first explored spatial distribution of FABP4/5 in atherosclerotic lesions in apolipoprotein E deficient (ApoE−/−) mice. FABP4 was only marginally detected in early and advanced lesions, whereas FABP5 was abundantly expressed in these lesions. In advanced lesions, the FABP5-positive area was mostly restricted to the foam cell layer adjacent to the lumen above collagen and elastic fibers with a high signal/noise ratio. Oil red O (ORO) staining revealed that FABP5-positive cells were lipid-rich in early and advanced lesions. Together, most of lipid-rich FABP5-positive cells reside adjacent to the lumen above collagen and elastic fibers. We next studied involvement of FABP5 in lesion formation of atherosclerosis using ApoE−/− FABP5−/− mice. However, deletion of FABP5 did not affect the development of atherosclerosis. These findings, along with previous reports, suggest a novel notion that FABP5 is a sensitive marker for bone marrow-derived lipid-rich macrophages in the luminal side of atherosclerotic lesions, although its functional significance remains elusive.

Key words: Atherosclerosis, Apolipoprotein E knockout mice, Foam cell, Oil red O staining

EARLY ASHHEROSCLEROTIC LESIONS

Early atherosclerotic lesions appear as fatty streaks consisting of lipid-rich foam cells.1-3 As atherosclerosis progresses, they become more complex lesions that are composed of multiple components such as lipid-laden macrophages, fibrous elements, extracellular cholesterol crystals, necrotic debris, chondrocyte-like cells and calcified plaques.4,5,6 It has been suggested that lipid-rich foam cells are developed through several possible scenarios. Among them, a response-to-retention hypothesis is widely accepted as the initiating process.7,8 It is also suggested that dendritic cells6,11 and proliferation of local macrophages6,12 are involved in foam cell formation. In advanced lesions, migration, proliferation and trans-differentiation of vascular smooth muscle cells (VSMCs) play a role in exacerbation of atherosclerosis, including formation of lipid-rich macrophage-like cells.5,6,13,14 Because advanced lesions are formed by quite complicated processes with multiple players, it is hard to identify each cell type and its origin in the lesions by conventional histological examination. In particular, it is hard...
to distinguish between BM-derived and VSMC-derived macrophages.

Apolipoprotein E deficient (ApoE−/−) mice exhibit atherosclerosis from early fatty streaks through advanced lesions containing multiple components, even under normal chow diet.5,12 In ApoE−/− mice, there are athero-prone arteries; the innominate artery is most prominent, followed by the aortic root, principal branches of the thoracic aorta, less curvature of the aortic arch, principal branches of the abdominal aorta, aortic bifurcation, iliac artery and pulmonary artery.16 Various stages of atherosclerosis are observed in an individual mouse at a point in time; this depends on the site of vessels. Thus, early and late stage atherosclerotic lesions are present at the same time in older ApoE−/− mice.

Fatty acid binding protein 4 (FABP4) and FABP5 are expressed in various cell types including adipocytes and macrophages and act as lipid chaperones to transport long chain FA into subcellular organelles.13,14 A series of studies with genetic and pharmacological inhibition of FABP 4/5 in mice have demonstrated that they play an important role in the development of insulin resistance, inflammation and atherosclerosis.19,20 In BM transplant experiments with FABP4/5-deficient cells, exacerbation of atherosclerotic lesions formation was suppressed in ApoE−/− and low-density lipoprotein receptor-deficient (LDLR−/−) mice, indicating that local expression of FABP4/5 in myeloid cells is involved in the development of atherosclerosis.19,20,24,25 However, it is still uncertain about the cell-type specificity and differential distribution between FABP4 and FABP5-expressing cells in early- and late-stage atherosclerotic lesions.

In this study, we explored spatial distribution of FABP4/5 in early and advanced atherosclerotic lesions in ApoE−/− mice with antibodies against them with high specificity.26 We found that FABP5 was strongly detected in lipid-rich foam cells in early and advanced atherosclerotic lesions, whereas FABP4 expression was modest at either stage. Although deletion of FABP5 did not affect the development of atherosclerosis in ApoE−/− mice, our histological findings suggest a possibility that FABP5 is a sensitive marker for newly formed foam cells that are probably derived from circulating myeloid cells.

Methods

The Institutional Animal Care and Use Committee (Gunma University Graduate School of Medicine) approved all studies. All experiments were performed in accordance with the NIH guidelines (Guide for the Care and Use of Laboratory Animals).

Mice: ApoE−/− mice with the C57BL6 background were provided by Professor Ishibashi, Jichi Medical University,29 FABP5−/− mice were generated as described previously.30

Double-deficient mice (ApoE−/− FABP5−/−) mice were generated from an intercross between these mice. Mice were housed in a temperature-controlled room with 12 hours light, 12 hours dark cycle and given unrestricted access to water and standard chow (CE-2, Clea Japan).

Qualitative estimation of FABP4/5 expression in atherosclerotic lesions: To estimate distribution of FABP4/5 expression in early and advanced atherosclerotic lesions, aortic tissues of female ApoE−/− mice were isolated at ages of 2, 4, 6 and 12 months after perfusion fixation with phosphate-buffered saline (PBS) containing 10% formalin (w/v) and embedded in paraffin. Serial sections were prepared from aortic sinuses, ascending and abdominal aortas.80 The sections were stained with hematoxylin and eosin (HE), Masson’s trichrome (MT), Elastica-van Gieson (E VG) and resorcin fuchsin (RF). Immunohistochemistry was performed with antibodies directed against FABP4,26 FABP5 (R&D, AF3077), F4/80 (AbD Serotec, A3-1), Mac-3 (BD Pharmingen, M3/84), Ki67 (Thermo Fisher Scientific, SP6), CD68 (SCB) and α-smooth muscle actin (SMA, DAKO) using the ABC kit (Vector Laboratories) according to the manufacturer’s protocol. Nuclei were counterstained with hematoxylin. Double immunofluorescence was carried out with first antibodies directed against FABP5, Mac-3, SMA and CD68 and secondary antibodies conjugated with Alexa Fluor-488 or -555 (Invitrogen). Nuclei were stained with 4’,6-diamino-2-phenylindole (DAPI, Wako Chemical). Images were obtained by Biorevo microscope (BZ-9000, Keyence, Japan).

Human sample and histological analysis: The study protocol was approved by the institutional review board of Gunma University hospital (approval no. 794). Carotid arteries obtained by autopsy were fixed with 4% paraformaldehyde and embedded in paraffin. Immunohistochemistry was performed as described earlier.

Relation between FABP expression and lipid accumulation: The hearts and aortas, perfused with PBS containing 10% formalin, were embedded in a Tissue-Tek O.C.T. compound (Sakura Finetek Co., Ltd., Tokyo). Six-micrometer-thick serial sections of aortic sinuses and aortas were cut using a cryostat (CM3050 S, Leica Biosystems, Germany) for Oil Red O stain (ORO, Sigma Aldrich) and immunohistochemistry with the FABP5 antibody.

Assessment of atherosclerosis in the whole aortas: After the aortas were excised and adventitial fat was removed, whole aortas were opened longitudinally from the aortic arch to the iliac bifurcation, mounted en face, and stained for lipids with Sudan IV (Tokyo Chemical Industry) as previously described.80 The extent of atherosclerotic areas was expressed as the percentage of the lesion area in the entire aortic surface area (WinROOF 2013, MITANI Co, Japan).

Imaging mass spectrometry

Tissue collection and sample preparation The mice were sacrificed and perfused with isotonic saline in the left cardiac ventricle followed by the removal of the aortic roots, embedding in 4% carboxymethyl cellulose sodium salt, and freezing in a hexane/dry ice bath. For imaging mass spectrometry (MS), the serial sections were mounted onto antistripping coated slides (S9901; Matsunami Glass, Osaka, Japan).

Desorption Electro spray Ionization-MS imaging The distribution of free fatty acids was observed using a Xevo G2 XS quadrupole-TOF mass spectrometer (Waters, Manchester, UK). Glass slides containing 10 μm slices were mounted on a stage and subjected to desorption electros-
spray ionization (DESI)-IMS in the negative ion mode over the mass range from $m/z$ 50 to 1200. The sprayer-to-surface distance was 1.0 mm; the sprayer to MS inlet distance was 5 mm; and the incident spray angle was set to 75°. The source parameters were adjusted to 3.5 kV capillary voltage, 120°C capillary temperature, and a nitrogen spray of 100 psi. To acquire DESI-MS images, tissues were raster-scanned at a velocity of 200 μm/s, with a scan time of 0.5 s and a spatial resolution of 100 μm. High Definition Imaging platform version 1.4 (Waters) was used to process the MS data and generate two-dimensional spatially resolved ion images.

**Results**

Aortic tissues of ApoE−/− mice were excised at ages of 2, 4, 6, and 12 months. Serial sections were prepared from aortic sinuses, ascending and abdominal aortas. HE staining revealed that atherosclerotic lesions were formed in 4-, 6-, and 12-month-old mice (Figure 1A and B) but not in 2-month-old ones (data not presented). Although thick complex atherosclerotic lesions (or advanced lesions) appeared more frequently in older mice, thin lesions (or early lesions) were also observed even at the age of 12 months (Figure 1A and B), depending on the preparation.

**Figure 1.** Immunohistochemical distribution of FABP4, FABP5 and F4/80 in atherosclerotic lesions in ApoE−/− mice: early (A) and advanced (B) lesions. Areas surrounded by the rectangle in HE staining are presented at higher magnification in lower panels. HE indicates hematoxylin eosin. Among more than 40 sections we estimated, the most representative photographs are presented.
Figure 2. A and B: Immunohistochemical distribution of FABP5 and Mac-3 in atherosclerotic lesions in ApoE-/- mice: early (A) and advanced (B) lesions. C and D: Immunofluorescent distribution of FABP5, Mac-3 and SMA in atherosclerotic lesions in ApoE-/- mice: early (C) and advanced (D) lesions. SMA indicates smooth muscle α-actin.

sites. Early lesions were mostly consisted of foam cells with clear cytoplasm, whereas thick lesions consisted of more complex components with a thin layer of foam cells adjacent to the lumen. Immunohistochemistry revealed
that FABP4 was marginally stained in both early and advanced lesions, whereas FABP5 was abundantly expressed (Figure 1A and B). In advanced lesions, the FABP5-positive area was nearly restricted to the foam cell layer adjacent to the lumen (Figure 1B). Expression of FABP4/5 was also confirmed in para-aortic adipose tissue (Figure 1A and B). F4/80, a representative macrophage marker, was marginally expressed in early lesions (Figure 1A). In advanced lesions, F4/80 staining was weak, unclear and broader than the foam cell layer detected by HE staining (Figure 1B). Expression of Mac-3, another macrophage marker, was modestly detected in early lesions (Figure 2A). In advanced lesions, Mac-3 was expressed not only in surface foam cell layers but also in complex lesions under the foam cell layer (Figure 2B and D). Double immunofluorescence revealed that expression intensity of FABP5 and Mac-3 was uneven in both early and advanced lesions although they mostly overlapped (Figure 2C and D). In addition, the signal/noise ratio of FABP5 was higher than that of Mac-3 (Figure 2A-D). FABP5 was not detected in SMA-positive cells (Figure 2C and D). Distribution of FABP4/5 expression in atherosclerotic lesions in ApoE⁻/⁻ mice is summarized in the Table. We further found that FABP5 was also detected in CD68-positive macrophages in human atherosclerotic lesions (Figure 3). Thus, it is likely that FABP5 is predominantly expressed in foam cells irrespective of atherosclerosis stages, suggesting that FABP5 is a possible and sensitive marker for foam cells located on the luminal side of atherosclerotic lesions.

Table. Distribution of the Positive Area for FABP4 and FABP5 in Atherosclerotic Lesions in ApoE⁻/⁻ Mice

| Early lesion | Advanced lesion | Adjacent to lumen | Adjacent to media |
|--------------|-----------------|-------------------|------------------|
| FABP4        | -               | -                 | ++               |
| FABP5        | ++              | ++                | -                |

Figure 3. Immunohistochemical (A–D) and immunofluorescent (E–G) distribution of FABP4, FABP5, CD68 and SMA in atherosclerotic lesions in the human carotid artery.
Figure 4. Relation between FABP5-positive cells and connective tissues in atherosclerotic lesions in ApoE−/− mice. Collagen fibers were detected using Masson’s trichrome (MT, blue) and Elastica-van Gieson (EVG, red) stains. Elastic fibers were detected using EVG (black) and resorcin fuchsin (RF, black) stains.

We next explored characteristics of FABP5-positive cells in combination with other staining methods. Most FABP5-positive cells were observed above collagen fibers detected by MT and EVG stains (Figure 4A-F) and elastic fibers detected by EVG and RF stains (Figure 4G-I). A few FABP5-positive cells were observed in complex lesions under foam cell layers (Figures 4D, 4G). These findings suggest that most FABP5-positive cells reside adjacent to the lumen and hardly infiltrate into complex lesions because of blocking by collagen and elastic fibers. Ki67, a mitosis marker, was expressed in FABP5-positive cells (Figure 5A), suggesting that some FABP5-positive cells undergo cell division. Oil red O (ORO) staining revealed that FABP5-positive cells are lipid-rich in both early and advanced lesions (Figure 5B). Altogether, most of lipid-rich FABP5-positive cells reside adjacent to the lumen above collagen and elastic fibers of complex lesions. These findings, along with previous reports, suggest a possibility that foamy BM-derived cells express FABP5 in nascent atherosclerotic lesions.

Strong expression of FABP5 in lipid-laden foam cell layers in atherosclerotic lesions allowed us to presume that FABP5 may promote the development of atherosclerotic lesions. To address the issue, ApoE−/− mice were bred with FABP5−/− to generate ApoE−/−FABP5−/−. After 6 months of feeding the mice standard chow diet, advanced lesions were confirmed in aortic sinus in ApoE−/− and ApoE−/−FABP5−/− mice without a significant difference of atherosclerosis degrees. Massive accumulation of lipid, assessed by ORO stains, was also detected even in ApoE−/− FABP5−/− mice (Figure 6A). En face preparation with Sudan IV stain revealed that lipid accumulation of aortas was equivalent between ApoE−/− and ApoE−/−FABP5−/− mice (Figure 6B). We further attempted to study whether FABP5 deletion affects contents of long-chain free FAs in atherosclerotic lesions using imaging MS. We found that the signal intensity of long-chain free FAs in lipid-rich lesions was much lower compared to those in other parts of the vessel structure and that FABP5 deficiency had a minor role in FA accumulation in atherosclerotic lesions (Figure
Figure 5. A: Immunohistochemical distribution of FABP5 and Ki67 in atherosclerotic lesions in ApoE-/- mice. B: Relation between FABP5-positive cells and lipid accumulation detected by oil red O (ORO) stains in atherosclerotic lesions in ApoE-/- mice. The foam cell layer was confirmed through an EVG stain.
Figure 6. Massive lipid accumulation in atherosclerotic lesions was not affected by FABP5 deletion in ApoE−/− mice. A: Serial sections of aortic roots were prepared from ApoE−/− and ApoE−/− FABP5−/− mice fed a standard chow diet for 6 months. B: En face preparation of aortas was stained with Sudan IV.

7). Thus, our data suggest that FABP5 deletion does not significantly affect the development of atherosclerosis in ApoE−/− mice under standard chow diet.

Discussion

In this study, we demonstrated a possibility that FABP5 can be a sensitive marker for lipid-rich macrophages, probably derived from myeloid cells during the formation of atherosclerotic plaque in ApoE−/− mice. FABP5 was abundantly and exclusively expressed in lipid-rich and Mac-3-positive foam cells in early and advanced lesions. Particularly, in advanced lesions, distribution of FABP5-positive cells was nearly restricted to a thin layer adjacent to the lumen, and collagen/elastic fibers were likely to block their infiltration into a deeper layer. These findings further suggest that FABP5-positive foam cells are recruited from vascular lumen and originate from circulating cells. Consistent with it, it is reported that in BM transplant experiments, GFP-positive myeloid cells are located in the surface layer, even in advanced atherosclerotic lesions.33 In contrast to distribution of FABP5, standard macrophage markers such as F4/80 and Mac-3 were expressed not only in foam cells but also in a wide area of
Accumulation of long-chain free FAs in lipid-rich lesions was estimated using imaging mass spectrometry. Location of the lipid-rich foam layer was confirmed using Masson’s trichrome (MT) and an oil red O (ORO) stain. The signal intensity of indicated FA surrounded by ovals was measured. The signal intensity ratio of DKO/WT in the foam cell layer for each long-chain free FA is presented on the right side of each panel. The experiments were performed twice. Note that the signal intensity of long-chain free FAs in lipid-rich lesions was much lower compared to that in other parts of the vessel structure. ROI indicates region of interest.
advanced lesions, suggesting that their specificity for foamy macrophages is not high enough. Besides, expression intensity of F4/80 and Mac-3 was obscure compared to that of FABP5. Thus, we suggest that histological detection of FABP5 can be a useful choice for identifying newly formed foam cells or lipid-laden macrophages that are probably derived from circulating myeloid cells. VSMCs have phenotypic plasticity and play a critical role in the development of advanced atherosclerotic lesions. They migrate from a medial layer, are oligoclonally expanded, and trans-differentiate into various types of cells in advanced lesions (i.e. macrophage-like cells, chondrocyte-like cells, mesodermal stem cells). During the process, they lose expression of smooth muscle-specific markers such as SM α-actin and myosin heavy chain while they display phenotypic conversion by expressing markers of other cell types, such as CD68, Mac-2 and Mac-3 for macrophages and SOX9 and RUNX2 for osteochondrogenic cells. Recent lineage tracing studies clearly demonstrated that lipid-rich macrophage-like cells are also derived from VSMC. Thus, it is hard to distinguish BM-derived macrophages from VSMC-derived macrophage-like cells by conventional histological examination. Although lineage tracing is a powerful tool for identifying the origin of cells, the technique cannot be readily performed in all laboratories working on atherosclerosis studies. As discussed earlier, FABP5 seems to be exclusively expressed in a nascent foam cell layer, probably derived from circulating myeloid cells, suggesting FABP5 as a sensitive marker for such cells. We suggest that detection of FABP5 may be a convenient way of distinguishing between BM-derived and VSMC-derived macrophages in atherosclerotic lesions for screening examination. This warrants exploration of whether only BM-derived macrophages express FABP5 in atherosclerotic lesions by lineage tracing and/or BM transplant in the future.

Babaev et al. reported that FABP5 deletion in macrophages suppresses atherosclerosis in LDLR−/− mice on a western-style hypercholesterolemic diet. In our study, however, we found no significant reduction in atherosclerosis in ApoE−/−FABP5−/− mice on a standard chow compared to ApoE−/− mice. These findings suggest that animal models of atherosclerosis (ApoE−/− versus LDLR−/−) and/or diet (standard chow versus western-style) affect atherosclerotic effects of FABP5. Notably, there are several articles demonstrating atherosclerotic effects of FABP4 and combined effects of FABP4/FABP5 in ApoE−/− mice, but there is no report regarding that of FABP5 in ApoE−/− mice. This suggests that ablation of FABP5 has a minor role in the development of atherosclerosis in ApoE−/− mice. Although expression levels of FABP5 in macrophages appear to be higher than those of FABP4 in histological analyses, atherosclerotic effects of FABP5 seem to be smaller than those of FABP4.

To find some mechanistic insights, we attempted to study how FABP5 is induced in macrophages in vitro. We prepared bone marrow-derived macrophages (BMDMs) from wild-type control and FABP5+/− mice. However, we failed to demonstrate induction of FABP5 expression by administration of several inflammatory stimuli such as lipopolysaccharide, tumor necrosis factor-α, interferon-γ and albumin-bound palmitic acid, even in control BMDM (data not presented). Besides, both BMDMs similarly responded to these inflammatory stimuli with comparable expression of inflammation-related genes (data not presented); this suggests that FABP5 is not involved in such ligand-mediated responses. Further studies are required to clarify mechanisms underlying induction of FABP5 in lipid-rich macrophages in atherosclerotic lesions.

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Disclosure

Conflicts of interest: None.

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