Atherosclerosis 269 (2018) 192–196

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Overexpression of perilipin1 protects against atheroma progression in apolipoprotein E knockout mice

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ARTICLE INFO

Article history:
Received 1 August 2017
Received in revised form
15 December 2017
Accepted 12 January 2018
Available online 17 January 2018

Keywords:
Perilipin1
PLIN1
Macrophage
Atherosclerosis

ABSTRACT

Background and aims: Perilipin1 (PLIN1), a lipid droplet-associated protein, plays an important role in the regulation of lipolysis and lipid storage in adipocytes. PLIN1 has recently been reported to be expressed in macrophages within atheroma plaques, suggesting PLIN1 may play a role in the accumulation of lipids at the arterial wall and in the development of atherosclerosis. To clarify the role of PLIN1 in the pathophysiology of atherosclerosis, we assessed the progression of atherosclerosis in *PLIN1* transgenic mice (*Plin1Tg*).

Methods: Plin1Tg were crossed with apolipoprotein E knockout mice (*ApoeKO*). C57BL/6J mice, *ApoeKO* and *Plin1Tg/ApoeKO* received a normal chow diet for 20 weeks. Body weight, gonadal fat mass and plasma lipid concentrations were measured. Aortas were collected for quantification of atheroma lesions and histological analysis by Oil Red O staining.

Results: Body weight, gonadal adipose mass and plasma triglyceride concentrations were not significantly different among the three groups. In contrast, the atherosclerotic lesion area was significantly increased in *ApoeKO* ($14.2 \pm 3.2\%$; $p < .01$) compared with C57BL/6J mice ($3.3 \pm 1.2\%$) and *Plin1Tg/ApoeKO* ($5.6 \pm 1.9\%$).

Conclusions: Overexpressed PLIN1 in macrophages had a protected role against atheroma progression in *ApoeKO* in the absence of changes in gonadal fat mass or plasma lipid levels, presumably due to modification of the stability and/or inflammatory profile of macrophages.

1. Introduction

Perilipin1 (PLIN1, perilipin A) is one of the lipid droplet-associated proteins that was originally identified in adipocytes [1]. In adipocytes, PLIN1 coats the lipid droplets and regulates both lipid storage and lipolysis. In the basal state, PLIN1 associates with the protein CGI-58, a coactivator of adipose triglyceride lipase (ATGL), and regulates the access of hormone-sensitive lipase (HSL) to lipid droplets, thus down-regulating basal lipolysis [2] [3]. Conversely, when adipocytes are stimulated by catecholamine, increased cAMP activates protein kinase A (PKA), resulting in the phosphorylation of HSL and PLIN1 in adipocytes [4]. Consequently, CGI-58 dissociates from phosphorylated PLIN1, and recruits phosphorylated HSL at the surface of the lipid droplets, thus activating lipolysis [5].

Following the discovery of PLIN1, a series of additional lipid droplet-associated proteins were identified. The perilipin family comprised five proteins: PLIN1, PLIN2 (adipose differentiation-related protein, adipophilin) [6], PLIN3 (tail-interacting protein 47, TIP47) [7], PLIN4 (S3-12) [8] [9], and PLIN5 (PAT1, LSPD5, OXPAT, MLDP) [10] [11] [12]. PLIN1 localizes generally in adipocytes and steroidogenic tissue. Under specific conditions, PLIN1 expression occurs also in macrophages, where it plays a crucial role in the progression of atherosclerosis [13]. However, the function of PLIN1 in macrophages is not yet known. Meanwhile, PLIN2 is the most abundant PAT protein in macrophage-derived foam cells. PLIN2 augments TNFα, MCP-1, and IL-6 expression in acetylated LDL-induced macrophages, suggesting that enhancing inflammation might be one role of PLIN2 in atherosclerosis [14].

Atherosclerosis is a chronic inflammatory disease that arises from an imbalance in lipid metabolism and a maladaptive immune

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https://doi.org/10.1016/j.atherosclerosis.2018.01.019

response driven by the accumulation of cholesterol-laden macrophages in the artery wall [15]. Macrophages play a principal role in atherosclerosis, having at least two different phenotypes: M1 and M2 macrophages, the former classically activated and the latter formed by alternative activation. A number of previous studies have demonstrated that M1 macrophages promote inflammation but M2 macrophages have anti-inflammatory properties [16].

We [17] previously reported the polarity of macrophages in human carotid artery plaques obtained by carotid endarterectomy. In that study, predominant infiltration of M1 macrophages was shown in probably-unstable plaque specimen. Conversely, in stable plaques from asymptomatic patients, M2 macrophages were dominantly observed despite the fact of less infiltration of total macrophage number compared with that in unstable plaques.

Following the observation above, we examined PLIN1 and PLIN2 expression in the plaques. In the stable plaques, PLIN1 expression was dominant compared with PLIN2 expression along with M2 macrophage ascendancy. In contrast, PLIN2 expression was prominent in unstable plaques in parallel with M1 macrophage infiltration. Therefore, we hypothesized that PLIN1/PLIN2 expression and macrophage polarity might be related to each other, consequently playing a role in the progression of atherosclerosis. We therefore conducted the present study to assess the effect of PLIN1 overexpression in macrophages on the progression of atherosclerosis in vivo using our *PLIN1* transgenic mice (*Plin1Tg*) [18].

2. Materials and methods

2.1. Antibodies

Polyclonal anti-PLIN1 antibody and anti-PLIN2 antibody were generated as previously described [2] [19]. Anti-CD68 antibody and anti-CD206 antibody were purchased from Abcam (Cambridge, England) Anti-CD11c antibody was purchased from Bio-Rad (Hercules, United States).

2.2. Animal experiments

Plin1Tg were generated using the aP2 promotor on a C57BL/6J background as previously described. The PLIN1 expression level in white adipose tissue in *Plin1Tg* was shown to be two times higher than that in control mice [18]. Apolipoprotein E knockout mice (*ApoeKO*) were purchased from the Jackson Laboratory (Bar Harbor, United States). C57BL/6J mice were purchased from Charles River Japan (Yokohama, Japan). *Plin1Tg* and *ApoeKO* were crossed to obtain *Plin1Tg/ApoeKO* mice. The mice were housed at the Graduate School of Medicine's Institute for Animal Experimentation at Hokkaido University in accordance with the institutional guidelines of Hokkaido University Graduate School of Medicine. All mice were housed at room temperature, maintained on a 12 h light/dark cycle, and given free access to water.

All mice received a normal chow diet (MF from Oriental Yeast, Tokyo, Japan) for 20 weeks. Body weight and gonadal fat mass were measured. Blood was collected from inferior vena cava and plasma separated by centrifugation for enzymatic determination (Wako, Tokyo, Japan; R&D Systems, Minneapolis, United States) of lipid concentrations and proinflammatory cytokine levels. Aortic sinuses and whole aortas were collected for quantification of atheroma lesions and histology.

2.3. Reverse transcription polymerase chain reaction (RT-PCR) analysis

Thioglycollate-elicited macrophages were isolated from C57BL/6J mice and *Plin1Tg* by washing the peritoneal cavity with 3 ml of phosphate-buffered saline one day after the mice were intraperitoneally injected with 50 μl of 4% thioglycollate in phosphate-buffered saline. Individual cell suspensions were washed with red blood cell lysis buffer (eBioscience, San Diego, United States). Total RNA was isolated from the isolated macrophages using an RNeasy Mini kit (QIAGEN, Venlo, Netherlands) according to the manufacturer's recommendations, and was used as the starting material for cDNA preparation. RT-PCR was performed using ReverTra-Plus (Toyobo, Osaka, Japan) in accordance with the manufacturer's protocols. Primer sequences are shown in Table 2 in Ref. [29].

2.4. Histology

Aortas were dissected from the aortic root to the iliac bifurcation, carefully separated from periarterial adipose tissue, and stained for lipid deposits with Oil Red O. The Oil Red O-positive areas were quantified and expressed as a percentage of total aorta area using a BZ-II Analyzer (Keyence). For histological studies, aortic sinuses were fixed in phosphate-buffered formalin and frozen before sectioning. Hematoxylin staining and immunocytology for whole macrophages (using anti-CD68 antibody), M1 macrophages (using anti-CD11c antibody), M2 macrophages (using anti-CD206 antibody), PLIN1 (using anti-PLIN1 antibody), and PLIN2 (using anti-PLIN2 antibody) were performed.

2.5. Statistical analysis

Results are expressed as means ± SD. The differences between the groups were assessed by un-paired *t*-test, analysis of variance, or Dunn's test. $p < .05$ was considered statistically significant. Data were analyzed using JMP Pro version 12.0.1.

3. Results

3.1. Biochemical markers did not differ between ApoeKO and Plin1Tg/ApoeKO

Thioglycollate-elicited peritoneal macrophages were isolated from C57BL/6J mice or *Plin1Tg*. Total RNA was prepared and analyzed by RT-PCR. Expressions of the *PLIN1* transgene and PLIN2 gene in the *Plin1Tg* macrophages was confirmed (Fig. 1). We next examined whether PLIN1 overexpression affected biochemical markers in *ApoeKO*. Body weight (C57BL/6J 26.6 ± 3.1 g; *ApoeKO* 29.0 ± 4.5 g; *Plin1Tg/ApoeKO* 27.5 ± 3.9 g) and gonadal fat mass

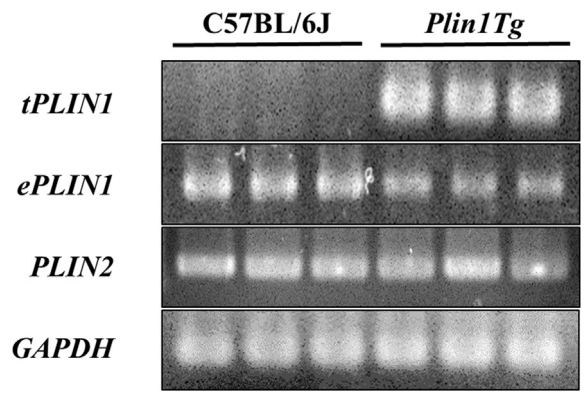

Fig. 1. Peritoneal macrophages were isolated from C57BL/6J mice and *PLIN1* transgenic mice (*Plin1Tg*), and mRNA was extracted.
(Left three lanes) Macrophages from C57BL/6J mice; (right three lanes) macrophages from *PLIN1Tg*. *tPLIN1* refers to the transgene induced in *PLIN1* transgenic mice. *ePLIN1* refers to the endogenous *PLIN1* gene in macrophages.

(C57BL/6J 356 ± 78 mg; *ApoeKO* 332 ± 124 mg; *Plin1Tg/ApoeKO* 424 ± 190 mg) were comparable among C57BL/6J mice, *ApoeKO* and *Plin1Tg/ApoeKO*. Plasma total cholesterol levels were significantly higher in *ApoeKO* (395 ± 80 mg) and in *PLIN1Tg/ApoeKO* (471 ± 138 mg) than in C57BL/6J mice (72 ± 11 mg), but there was no significant difference between *ApoeKO* and *Plin1Tg/ApoeKO*. Plasma TNFa was undetectable in all groups. Plasma IL-6 levels (C57BL/6J 22.2 ± 14.1 pg/ml; *ApoeKO* 64.2 ± 37.4 pg/ml; *Plin1Tg/ApoeKO* 28.2 ± 31.1 pg/ml) tended to be high in *ApoeKO*, but did not differ significantly between *ApoeKO* and *Plin1Tg/ApoeKO* ($p = .069$) (Table 1).

3.2. Overexpression of PLIN1 suppressed atherosclerosis development in ApoeKO

We examined the size of the atherosclerotic lesions in the aortic sinus area (Fig. 2) and in the whole aorta using an en face method (Fig. 3A) with Oil Red O staining. The lesions were quantified as a percentage of total aorta area (Fig. 3B). Wild type mice had almost no atherosclerotic lesions (3.3 ± 1.0%), whereas a lack of apolipoprotein E resulted in an increase in lesion size (14.2 ± 0.9%). Interestingly, the atherosclerotic lesion size was significantly decreased in *Plin1Tg/ApoeKO* compared with *ApoeKO* (5.6 ± 0.7%, $p < .01$) despite the comparable body weight, visceral fat and plasma lipid levels in the two strains. Therefore, PLIN1 overexpression protects against atherosclerosis development in *ApoeKO*.

3.3. Immunohistological analysis

We next assessed the polarities of the infiltrated macrophages and the expression of PLINs in the plaques (Fig. 4). In wild-type mice, there were almost no plaques. Neither macrophage infiltration nor expression of PLINs was evident. Conversely, both *ApoeKO* and *Plin1Tg/ApoeKO* exhibited plaques with massive macrophage infiltration. The expression level of CD11c, an M1 macrophage marker, showed higher intensity in *ApoeKO* than in *Plin1Tg/ApoeKO*, whereas no obvious difference in the expression level of the M2 macrophage marker CD206 was observed between the strains. PLIN1 expression was clearly identified in *Plin1Tg/ApoeKO*, but was not detected in *ApoeKO*. Conversely, PLIN2 expression was observed in both *ApoeKO* and *Plin1Tg/ApoeKO*, and its levels were obviously higher in *ApoeKO* than in *Plin1Tg/ApoeKO*.

Table 1
Biochemical markers.

	C57BL/6J	*ApoeKO*	*Plin1Tg/ApoeKO*
Body weight (g)	26.6 ± 3.1	29.0 ± 4.5	27.5 ± 3.9
Gonadal fat (mg)	356 ± 78	332 ± 124	424 ± 190
Triglyceride (mg/dl)	69 ± 25	99 ± 43	120 ± 71
Total cholesterol (mg/dl)	72 ± 11	395 ± 80*	471 ± 138*
IL-6 (pg/ml)	22.2 ± 14.1	64.2 ± 37.4	28.2 ± 31.1

*$p < .01$ *vs.* C57BL/6J.

4. Discussion

In this study, we explored the effect of PLIN1 overexpression on atherosclerosis progression. Our results support an atheroprotective role of PLIN1 by the observation that the extent of atheroma lesions was decreased in ApoE knockout mice overexpressing PLIN1 compared with in standard ApoE knockout mice. PLIN1 is expressed only in adipocytes and macrophages, and ordinary risk factors such as body weight, gonadal fat mass, plasma lipid levels, were not altered by PLIN1 overexpression at least in younger *Plin1Tg*. Accordingly, the atheroprotective property of PLIN1 was likely to be related with the effects of PLIN1 on macrophages. Similar information regarding atheroprotective role of Plin1 were recently published by Langlois et al. [20]. In their study, the extent of atheroma lesions was increased in LDL receptor knockout mice with *PLIN1* ablated. However, we can see major limitations in the interpretation of their study. Body weight and fat mass were highly reduced by PLIN1 ablation, thus the effect of *PLIN1* ablation on atheroma could include such “indirect effect” of PLIN1, apart from “direct” interaction of PLN1 with macrophages. Our study has, not only supported the atheroprotective role of PLIN1 proven by the previous studies, but also clarified the direct effect of PLIN1 on macrophages for the first time.

In addition, we found that PLIN1 overexpression was correlated with a change in macrophage polarity in plaques; explaining in part the decrease in atherosclerosis. The balance of macrophages in plaques is dynamic and both macrophage numbers and the inflammatory phenotype influence plaque fate [15]. PLIN1 has been detected in THP-1 cells and human monocyte-derived macrophages, but its role in regulating foam cell formation remains controversial [13] [21] [22]. PLIN2 is the most abundant PAT protein in human and mouse macrophage-derived foam cells [23] [24] [25], and augments tumor necrosis factor-alpha (TNFα), monocyte chemoattractant protein-1 (MCP-1), and interleukin 6 (IL-6) induction in acetylated LDL-induced macrophages. Those observations have suggested that enhancing inflammation might be one role of PLIN2 in atherosclerosis [14]. The hypothesis is supported by the study

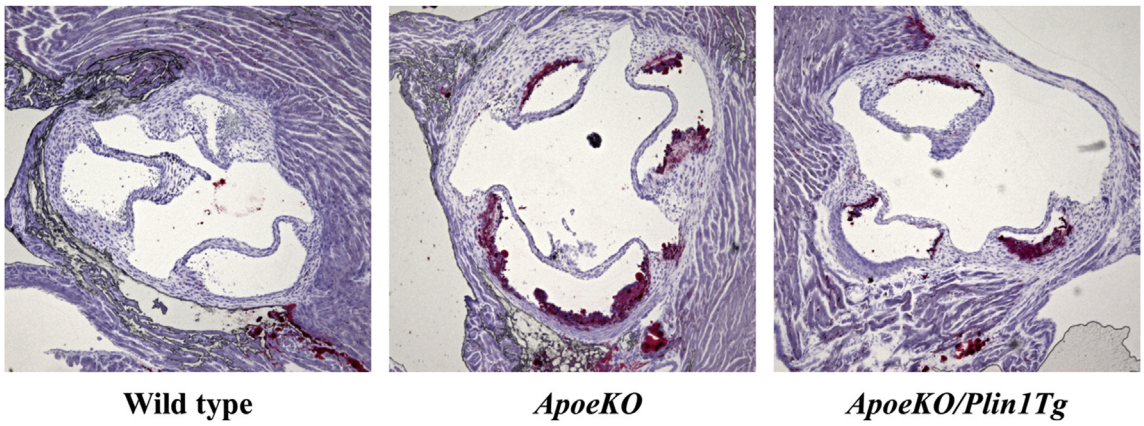

Fig. 2. Representative sections of atherosclerotic lesions stained with Oil Red O in aortic sinuses isolated from wild-type mice, apolipoprotein E knockout mice (*ApoeKO*) and *Plin1Tg/ApoeKO*.

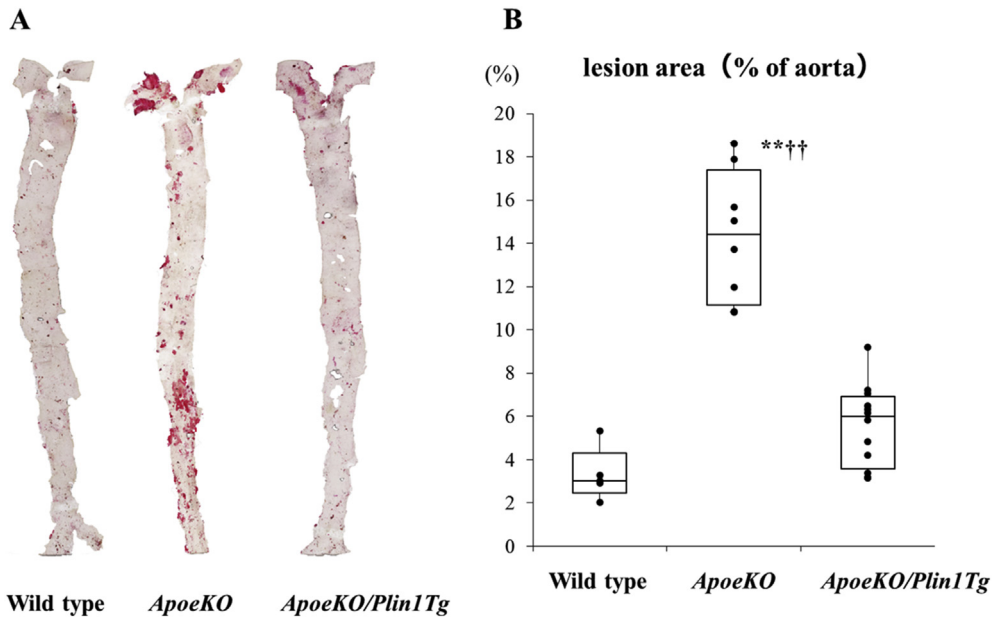

Fig. 3. Atherosclerotic lesions and atheroma.
(A) Representative sections of atherosclerotic lesions in aorta isolated from wild type mice, *ApoeKO* and *Plin1Tg/ApoeKO* and stained with Oil Red O. (B) Quantification of atheroma in aortas of wild-type mice, *ApoeKO* and *Plin1Tg/ApoeKO*. Atheroma was quantified via Oil Red O staining of lipid deposits (en face method). Results are shown as individual values and box-and-whisker plots. $^{**}p < .01$ *vs*. wild-type. $\ddagger p < .01$ *vs*. *Plin1Tg/ApoeKO*.

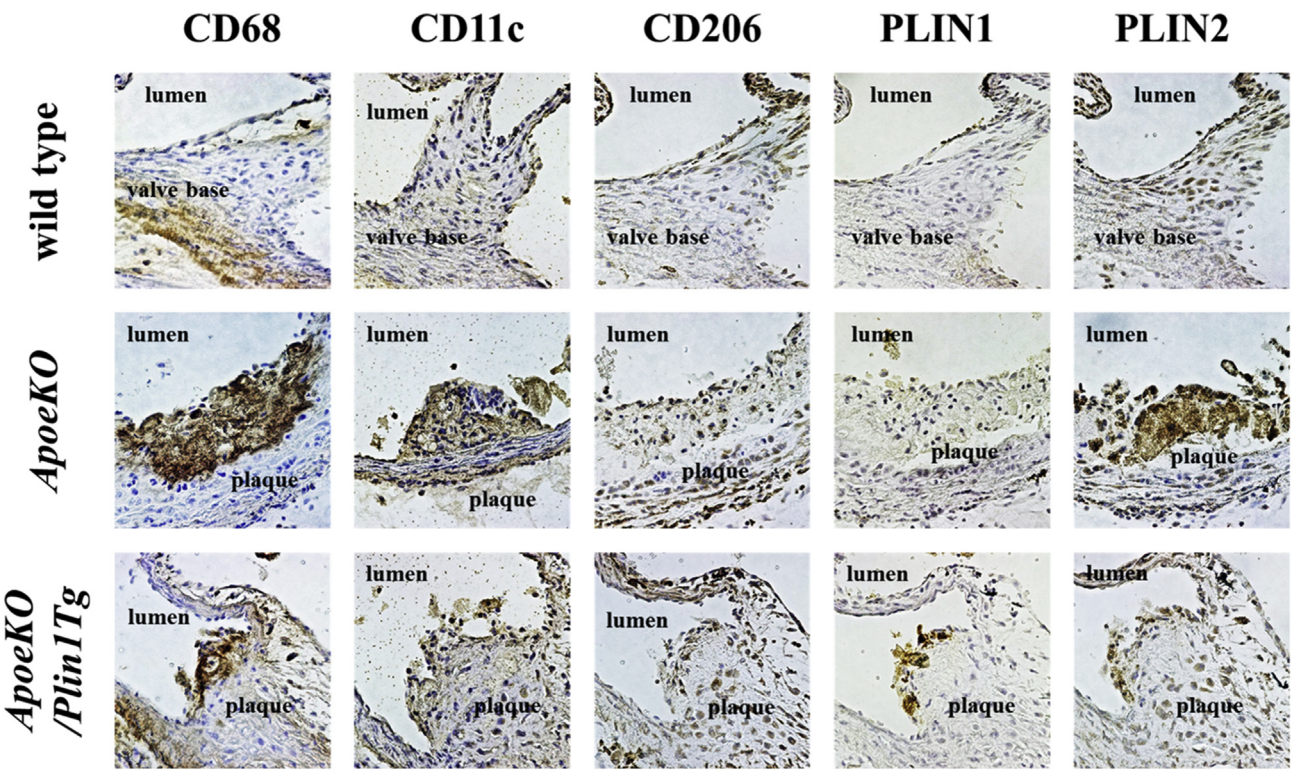

Fig. 4. Macrophage infiltration and expression of lipid droplet-associated proteins in plaques.
Frozen sections of aortic sinuses were stained with hematoxylin and anti-CD68 (macrophages), anti-CD11c (M1 macrophages), anti-CD206 (M2 macrophages), anti-PLIN1 (PLIN1) or anti-PLIN2 (PLIN2) antibody. Representative sections of atheroma plaques (*ApoeKO* and *Plin1Tg/ApoeKO*) or aortic valve base (wild-type mice) are shown.

that *PLIN2* gene inactivation in *ApoeKO* significantly reduced the number of lipid droplets in the foam cells in atherosclerotic lesions and protected mice against atherosclerosis [25].

The close relationship between PLIN1 and PLIN2 in adipocytes has been well established. During normal cultured adipocyte differentiation, PLIN2 expression occurs first, then after several days of culture PLIN1 reciprocates PLIN2 expression. However, during *PLIN1*-null adipocyte differentiation, PLIN2 expression remains

constant [4]. In *PLIN1* knockout mice, PLIN2, rather than PLIN1, coats the surface of lipid droplets in adipocytes [26]. It is likely that the presence of PLIN2 protein reflects the lack of PLIN2 displacement by PLIN1 in the PLIN1 null animals.

However, a different interaction between PLIN1 and PLIN2 in macrophages is possible. PLIN1 overexpression did not decrease the PLIN2 expression level noticeably in cultured macrophages derived from human monocytes (data not shown) or in peritoneal macrophages. There were no significant differences among the three groups in proinflammatory cytokine mRNA expression in adipose tissue (data not shown), and there was also no significant difference in plasma IL-6 concentration between *ApoeKO* and *Plin1Tg/ApoeKO*. Therefore, we believe that PLIN1 overexpression might induce the atheroprotective effect in *Plin1Tg* directly, rather than via changes in PLIN2 expression. However, to confirm that PLIN1 overexpression by itself influences atherosclerosis outcome, additional investigation should be performed in a *PLIN2* knockout background.

PLIN1 stably stores triglycerides in lipid droplets in adipocytes in the basal state. Compared with PLIN1, PLIN2 cannot store lipids effectively and induces continuous low-level lipolysis without PKA stimulation. Thus, increased lipolysis and diminished adipocyte size were observed in *PLIN1* knockout mice [27]. During macrophage-derived foam cell formation, PLIN2 is normally located on cytoplasmic lipid droplets [28]. When an excess volume of lipids flows into a macrophage, some degree of lipolysis is sustained because the lipid storage ability of PLIN2 is lower than that of PLIN1, resulting in more fatty acid and cholesterol accumulating inside and possibly also around the macrophage. If PLIN1 is highly expressed compared with PLIN2 in macrophages, triglyceride and cholesterol ester would be stably stored in lipid droplets and fatty acid production via lipolysis would be suppressed, thus leading to the suppression of inflammation in plaques. Further study will clarify these mechanisms.

In conclusion, overexpression of PLIN1 in macrophages protected against atheroma progression in the absence of changes in common risk factors. Changes in PLIN1 expression on lipid droplets in macrophages may play a role in the progression of atherosclerosis by modifying lipid droplet stability and subsequently influencing macrophage polarity and inflammation.

Conflict of interest

The authors declare they do not have anything to disclose regarding conflicts of interest with respect to this manuscript.

Author contributions

H Miyoshi designed the study. K Yamamoto performed the experiments and wrote the article. All authors participated in the analysis and interpretation of the data.

Acknowledgements

We thank N Fujimori and M Watanabe for technical assistance. HM thanks Dr. James W. Perfield II (University of Missouri, MO, USA) for his continued support and mentorship.

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