Regular Article

Acyl Chain Preference in Foam Cell Formation from Mouse Peritoneal Macrophages

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Received July 27, 2017; accepted October 18, 2017

Macrophage foam cells play critical roles in the initiation and development of atherosclerosis by synthesizing and accumulating cholesteryl ester (CE) in lipid droplets. However, in analyzing lipid metabolism in foam cell formation, studies have focused on the sterol group, and little research has been done on the acyl chains. Therefore, we adapted a model system using liposomes containing particular acyl chains and examined the effect of various acyl chains on foam cell formation. Of the phosphatidylserine (PS) liposomes tested containing PS, phosphatidylcholine, and cholesterol, we found that unsaturated (C18:1), but not saturated (C16:0 and C18:0), PS liposomes induced lipid droplet formation, indicating that foam cell formation depends on the nature of the acyl chain of the PS liposomes. Experiments on the uptake and accumulation of cholesterol from liposomes by adding [¹⁴C]cholesterol suggested that foam cell formation could be induced only when cholesterol was converted to CE in the case of C18:1 PS liposomes. Both microscopic observations and metabolic analysis suggest that cholesterol incorporated into either C16:0 or C18:0 PS liposomes may stay intact after being taken in by endosomes. The [¹⁴C]C18:1 fatty acyl chain in the C18:1 PS liposome was used to synthesize CE and triacylglycerol (TG). Interestingly, the [¹⁴C]C16:0 in the C18:1 PS liposome was metabolized to sphingomyelin rather than being incorporated into either CE or TG, which could be because of enzymatic acyl chain selectivity. In conclusion, our results indicate that the acyl chain preference of macrophages could have some impact on their progression to foam cells.

Key words foam cell formation; liposome; acyl chain preference; cholesteryl ester accumulation; mouse peritoneal macrophage; sphingomyelin

Brown and Goldstein first described that the scavenger receptors on macrophages are a family of cell surface glycoproteins that recognize and uptake modified low-density lipoproteins (LDL), such as oxidized LDL and acetylated LDL, in 1979.¹⁾ Subsequent cloning has revealed that the scavenger receptors are divided into eight distinct classes termed A through H, on the basis of amino acid sequence comparisons and domain architecture.²⁾ In macrophages, exogenous cholesterol and cholesteryl ester (CE) are incorporated into the cells from circulating LDL through receptor-mediated endocytosis,³⁾ and delivered from early endosomes to late endosomes/lysosomes. After CEs are hydrolyzed by acidic lipases to free cholesterol and fatty acids, free cholesterol is transferred from the endosome/lysosome to the endoplasmic reticulum (ER), plasma membrane, and other cellular compartments by the Niemann–Pick C1 (NPC1) protein.^{4,5)} In NPC1-deficient cells, free cholesterol accumulates in late endosomes/lysosomes. Nishikawa *et al.* studied the mechanism of macrophage recognition and the metabolism of membrane lipids with exposed acidic phospholipids. They showed that liposomes containing cholesterol and acidic phospholipids, especially phosphatidylserine (PS), were taken up by macrophages, and then cholesterol was esterified. Macrophages accumulate lipid droplets after the uptake of modified LDL, becoming foam cells, and it has been demonstrated that this process occurs via scavenger receptors.⁶⁾ Nishikawa *et al.* also reported that radiolabeled cellular fatty acyl chains of phospholipids were incorporated into neutral lipid fractions, such as CEs and triacylglycerols (TG), upon the incubation of liposomes with

macrophages.⁷⁾ Selective incorporation of the acyl chains into neutral lipids was observed, for example oleate (C18:1) was preferentially incorporated into the CE fraction. In contrast, linoleate (C18:2) and arachidonate (C20:4) were predominantly incorporated into the TG fraction. These results are consistent with the fatty acid profile of CEs accumulated in atherosclerotic plaques, which is predominantly C18:1. Based on the fact that the major components of fatty acids in LDL CEs are C18:1 and C18:2,⁸⁾ the acyl chains may be used selectively in macrophage foam cell formation, and may play an important role in pathology.

Measurement of cholesterol and CEs is performed using a variety of techniques. The traditional method is to use TLC.^{9,10)} An alternative technique is HPLC coupled with various types of detectors, such as UV or light scattering detection.^{11–16)} GC-MS is another technique.¹⁷⁾ LC/MS has recently become more popular as a useful tool to detect cholesterol and CEs.^{18–24)} However, as for the accumulation of CE, studies have focused on the metabolism of the sterol moiety and little research has been done on the acyl part even though macrophage foam cells play critical roles in the initiation and development of atherosclerosis. The model system presented here, using PS-containing liposomes, has the advantage that phospholipids containing specific acyl chains can be used. In the present study, we focused on the effect of a variety of different acyl chains on the macrophage synthesis of CEs and the formation of foam cells, which is the first step in atherosclerosis.

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MATERIALS AND METHODS

Reagents 1,2-Dipalmitoyl-phosphatidylcholine (PC) (1,2-C16:0 PC), 1,2-dipalmitoyl-PS (1,2-C16:0 PS), 1,2-distearoyl-PC (1,2-C18:0 PC), 1,2-distearoyl-PS (1,2-C18:0 PS), 1,2-dioleoyl-PC (1,2-C18:1 PC), and 1,2-dioleoyl-PS (1,2-C18:1 PS) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, U.S.A.). 1,2-Di[1-¹⁴C]palmitoyl-glycerophosphocholine and 1,2-di[1-¹⁴C]oleoyl-glycerophosphocholine were purchased from PerkinElmer, Inc. (Billerica, MA, U.S.A.). [4-¹⁴C]Cholesterol, [1-¹⁴C]palmitic acid, [1-¹⁴C]oleic acid, and [1-¹⁴C]linoleic acid were purchased from GE Healthcare (Little Chalfont, U.K.). Cholesterol, lipoprotein-deficient serum, and Oil Red O were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Cells Five- to six-month-old retired female ICR mice were obtained from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). Mouse peritoneal macrophages were prepared as described previously.⁶⁾ Peritoneal cells were harvested from unstimulated mice using phosphate buffered saline (PBS). The cells were collected by centrifugation (400×*g*, 5 min, 4°C) and washed once with Dulbecco's modified Eagle's medium (DMEM). The cells were resuspended at 2×10⁶ cells/mL in DMEM, and aliquots (1 mL) were dispensed into a 12-well plastic microplate. After incubation for 2 h in a humidified CO₂ (5%) atmosphere at 37°C, cells were rinsed three times with DMEM, and the adherent cells were incubated overnight in DMEM. In the metabolic labeling experiment, the cells were radiolabeled by adding [1-¹⁴C]C16:0 (0.1 μCi/mL) or [1-¹⁴C]C18:1 (0.1 μCi/mL) to the medium. After overnight incubation, the cells were rinsed three times with DMEM to remove detached cells. Then, the medium was replaced with 1 mL of DMEM with 6% lipoprotein-deficient serum containing liposomes, and the cells were cultured for the indicated times for each experiment. In the experiment investigating the metabolism of liposome constituents, the indicated radiolabeled liposomes were used as shown in Table 1 and Figs. 2–4. This study was conducted according to the guidelines of, and approved by, the Experimental Animal Ethics Committee of Teikyo University (approval #12-050).

Preparation of Liposomes Liposomes were prepared as described previously.²⁵⁾ Lipid mixture was placed in a

glass test tube, and the organic solvent was dried under nitrogen gas. The dried lipids were dispersed with a vortex mixer solution and an ultrasonic bath in DMEM (50 μL/well). PS liposomes were composed of PC–PS–cholesterol=100:100:210 nmol/well. For radiolabeled liposomes, [4-¹⁴C]cholesterol (0.1 μCi/1.9 nmol/mL), 1,2-di[1-¹⁴C]C16:0-glycerophosphocholine (0.1 μCi/0.91 nmol/mL), or 1,2-di[1-¹⁴C]C18:1-glycerophosphocholine (0.1 μCi/0.90 nmol/mL) were added to the lipid mixture followed by the same procedure as described above. The liposomes used in the present study are listed in Table 1. The liposome sizes were measured using an electrophoretic light scattering spectrophotometer (ELS-Z, Otsuka Electronics, Osaka, Japan).

Oil Red O Staining The Oil Red O solution was prepared by mixing three parts of stock solution (0.5% Oil Red O in isopropanol) and two parts of water, followed by incubation for 10–30 min at room temperature and filtration through a 0.2 μm filter. Cells were washed three times with PBS, and fixed with 500 μL of 3.7% formaldehyde. Fixed cells were then incubated with 500 μL of 60% isopropanol for 10 min, and stained with the Oil Red O solution for 10–30 min.⁶⁾

Lipid Analysis Macrophages were lysed in Tris–HCl (pH 7.4) with 0.1% sodium dodecyl sulfate. Total lipids were extracted by the Bligh and Dyer method.²⁶⁾ The organic phase was collected and dried under nitrogen gas. The extracted lipids were reconstituted with chloroform–methanol (1:1), and separated by TLC. Neutral lipids were analyzed by one-dimensional TLC in which *n*-hexane–diethyl ether–acetic acid (70:30:1) was used as the solvent. Phospholipids were analyzed by two-dimensional TLC in which methanol–chloroform–90% formic acid (25:65:10) and methanol–chloroform–water–ammonia water (40:50:3:7) were used as the solvents for the first and second dimensions, respectively.²⁷⁾ Dried TLC plates were exposed to imaging plates, and the radioactivity was quantified by BAS-1500 (FUJIFILM, Tokyo, Japan). A known amount of [4-¹⁴C]cholesterol was spotted on the corner of each plate, and used as the standard for calculations.

RESULTS

Acyl Chain Preference of PS Liposomes in Lipid Droplet Formation in Mouse Peritoneal Macrophages

We used

Table 1. Composition of the Liposomes Used in This Study

Relevant figures	Liposomes name	Phosphatidylserine	Phosphatidylcholine	Cholesterol
1	C16:0 PS	1,2-C16:0 PS	1,2-C16:0 PC	Cholesterol
1	C18:0 PS	1,2-C18:0 PS	1,2-C18:0 PC	Cholesterol
1	C18:1 PS	1,2-C18:1 PS	1,2-C18:1 PC	Cholesterol
1	C18:1 PC	—	1,2-C18:1 PC	Cholesterol
1	Natural PS	Bovine brain PS	Egg PC	Cholesterol
2, 3	C16:0 PS*	1,2-C16:0 PS	1,2-C16:0 PC	[4- ¹⁴ C]Cholesterol
2	C18:0 PS*	1,2-C18:0 PS	1,2-C18:0 PC	[4- ¹⁴ C]Cholesterol
2, 3	C18:1 PS*	1,2-C18:1 PS	1,2-C18:1 PC	[4- ¹⁴ C]Cholesterol
2	C18:1 PC*	—	1,2-C18:1 PC	[4- ¹⁴ C]Cholesterol
3	C16:0 PS/C18:1 PC*	1,2-C16:0 PS	1,2-C18:1 PC	[4- ¹⁴ C]Cholesterol
3	C18:1 PS/C16:0 PC*	1,2-C18:1 PS	1,2-C16:0 PC	[4- ¹⁴ C]Cholesterol
4	C16:0 PS (C16:0*)	1,2-C16:0 PS	1,2-[¹⁴ C]C16:0 PC	Cholesterol
4	C16:0 PS (C18:1*)	1,2-C16:0 PS	1,2-C16:0 PC+1,2-[¹⁴ C]C18:1 PC (trace)	Cholesterol
4	C18:1 PS (C16:0*)	1,2-C18:1 PS	1,2-C18:1 PC+1,2-[¹⁴ C]C16:0 PC (trace)	Cholesterol
4	C18:1 PS (C18:1*)	1,2-C18:1 PS	1,2-[¹⁴ C]C18:1 PC	Cholesterol

Fig. 1. Light Microscopy of Liposomes Stained with Oil Red O

(A) C16:0 PS liposomes, (B) C18:0 PS liposomes, (C) C18:1 PC liposomes, (D) C18:1 PS liposomes, and (E) natural PS liposomes were added to mouse peritoneal macrophages, and the cells were cultured for 5 d before staining with Oil Red O.

synthetic phospholipids with various fatty acids to prepare PS liposomes composed of PS, PC, and cholesterol, and examined subsequent foam cell formation. Neither C16:0 PS liposomes nor C18:0 PS liposomes induced lipid droplet formation (Figs. 1A, B). However, C18:1 PS liposomes induced the formation of lipid droplets (Fig. 1C). C18:1 PC liposomes did not induce lipid droplet formation, suggesting that addition of PS is required for lipid droplet formation (Fig. 1D). Natural PS liposomes induced foam cell formation (Fig. 1E), which is consistent with the results of Nishikawa *et al.*, in which the addition of PS, purified from bovine brains, into liposomes induced foam cell formation.⁶⁾ Neither C16:0 PC liposomes nor C18:0 PC liposomes induced lipid droplet formation (data not shown). Foam cells were detected by Oil Red O staining of intracellular lipid droplets. These results suggest that macrophages may have a fatty acid preference for the uptake of liposomes during foam cell formation.

Effect of the Acyl Chain Species in Liposomes on the Accumulation of CE Next, we examined the acyl group preference in the cellular uptake and esterification of cholesterol using radiolabeled cholesterol. We found that PS liposomes with saturated fatty acids, such as C16:0 or C18:0, increased the amount of cholesterol incorporated into cells in a time-dependent manner; however, cholesterol was not converted to CE and remained as cholesterol (Figs. 2A, B). On the other hand, [¹⁴C]cholesterol was incorporated in C18:1 PS* liposomes, and the cholesterol incorporated into cells was converted to CE (Fig. 2C). This result differs from that obtained with C16:0 or C18:0 PS liposomes, indicating that lipid droplet formation leading to foam cell formation might be induced only when cholesterol is converted to CE, and also suggests that this esterification process may depend on the fatty acid species composing the liposomes. Liposomes composed of C18:1 PC and cholesterol without PS did not induce the incorporation of cholesterol or the synthesis of CE (Fig. 2D).

Fig. 2. Cholesterol Metabolism in Liposomes

[¹⁴C]Cholesterol was added when preparing liposomes with cholesterol and synthetic phospholipids. After the cells were cultured for the indicated time period, they were collected and lipids were extracted by the Bligh and Dyer method. Subsequently, neutral lipids were separated by TLC, and the radioactivity (mean $\pm$ S.D., $n=4$) measured using an image analyzer and expressed as dpm per μ g of total cell protein. Open circles represent cholesterol. Filled circles represent cholesteryl ester. (A) C16:0 PS* liposomes, (B) C18:0 PS* liposomes, (C) C18:1 PS* liposomes, and (D) C18:1 PC* liposomes.

Next, we used PS liposomes containing PS, PC, and cholesterol, in which either the PS or PC of the C18:1 acyl chain was replaced with C16:0. Both C16:0 PS/C18:1 PC* and C18:1 PS/C16:0 PC* liposomes increased the incorporation of [¹⁴C]-cholesterol compared with C16:0 PS* liposomes (Fig. 3).

Fig. 3. Effects of Acyl Chain Length and Saturation of Phospholipids in Liposomes

PS liposomes were generated with different compositions of the acyl chains of PC and PS. Trace amounts of [^{14}C]cholesterol were additionally added to the liposomes. After mouse peritoneal macrophages were incubated with the prepared liposomes for 5 d, lipids were extracted, and the radioactivity (average $\pm$ max/min, $n=2-6$) was measured as described in Fig. 2. White bars indicate the incorporation of cholesterol, and black bars indicate the synthesis of CE. The constituents of each liposome are described in Table 1.

However, neither C16:0 PS/C18:1 PC* liposomes nor C18:1 PS/C16:0 PC* liposomes produced as much [^{14}C]CE as C18:1 PS* liposomes. These results suggest that the esterification of cholesterol is induced by C18:1 in either PC or PS. Thus, liposomal phospholipid fatty acyl chains may influence the synthesis of CE, lipid droplet formation, and foam cell formation. Therefore, we next analyzed the metabolism of the fatty acyl chain of liposomal phospholipids by adding radioisotope labeled PC to PS liposomes as a tracer.

Metabolism of Liposome-Derived Acyl Chains during Foam Cell Formation When [^{14}C]C16:0 PC was added to C16:0 PS liposomes (C16:0 PS (C16:0*) liposomes) under conditions in which foam cell formation does not occur, 71% of the radioisotope remained bound to PC and was not metabolized to form CEs or TG (Figs. 4A, B) after 5 d of incubation. Similarly, when [^{14}C]C18:1 PC was added to C16:0 PS liposomes (C16:0 PS (C18:1*) liposomes), 54% of the radiolabeled C18:1 remained as PC (Fig. 4C), meaning that more than half of the fatty acids were not metabolized to CEs or TG. Interestingly, when [^{14}C]C16:0 PC was added to C18:1 PS liposomes (C18:1 PS (C16:0*) liposomes), the radiolabeled C16:0 was metabolized to form sphingomyelin (SM), rather than being incorporated into CEs or TG during foam cell formation (Fig. 4D). In contrast, when [^{14}C]C18:1 PC was added to C18:1 PS liposomes (C18:1 PS (C18:1*) liposomes) under conditions for foam cell formation, 87% of the fatty acids in the radiolabeled C18:1 were released from PC, metabolized to CEs (40%) and TG (23%), and considered to be accumulated in the lipid droplets (Fig. 4E).

DISCUSSION

In the analysis of lipid metabolism in foam cell formation, the majority of studies have focused on the sterol group, and little attention has been paid to the acyl group. Nishikawa *et al.* reported that PS liposomes were recognized by the scavenger receptors on mouse peritoneal macrophages and effectively metabolized. Consequently, the cells showed the typical feature of lipid droplets that accumulate a large amount of

Fig. 4. Metabolism of Phospholipid Acyl Chains in Liposomes

Two-dimensional radioactivity distribution of C16:0 PS (C16:0*) liposome (A). C16:0 PS liposomes (B, C) and C18:1 PS liposomes (D, E) were prepared with trace amounts of radioisotope-labeled phospholipids, [^{14}C]C16:0 PC (B, D) and [^{14}C]C18:1 PC (C, E). Both liposomes contained cholesterol. After mouse peritoneal macrophages were incubated with the prepared liposomes for 5 d, the lipids were extracted. Neutral lipids and phospholipids were separated by 1-dimensional or 2-dimensional TLC, and the radioactivity was measured using an image analyzer (A–D), $n=3$.

CEs and TG in the cytosol.⁶⁾ In the present study, we used liposomes with various synthetic phospholipids to further examine the effect of liposomal phospholipid acyl chains on the esterification of cholesterol. The results we obtained suggest that membrane fluidity, enzymatic selectivity, and the size of the liposomes are involved in the uptake of liposomes, the esterification of cholesterol, and the synthesis of SM.

First, the fluidity of the liposomal membrane could affect the release of cholesterol from the liposomes in the late endosomal compartments or lysosomes. Nakagawa *et al.* have reported previously that liposomal cholesterol can transfer between co-incubated liposomes having different mole fractions of cholesterol.²⁸⁾ They also found that cholesterol derived from “solid” liposomes with saturated phospholipids is unlikely to be transferred to other liposomal membranes compared with “unsolid” liposomes with unsaturated phospholipids. These findings support a possible explanation for our results using C16:0 PS and C18:0 PS. After PS liposomes with unsaturated phospholipids were incorporated into macrophages and delivered to late endosomes/lysosomes, the acyl chain in PC/PS

could be cleaved by phospholipase A₁/A₂ (PLA₁/A₂).²⁹⁾ As a result, cholesterol would be able to egress from unsaturated “unsolid” liposomes, and would be transferred from the endosome/lysosome to the ER. However, in case of saturated PS liposomes, cholesterol might not egress efficiently from “solid” liposomes causing attenuation of the acylation of cholesterol. Our data suggest that the esterification of cholesterol is induced by C18:1 in either PC or PS (Fig. 3). Interestingly, the results show some differences in the incorporation of [¹⁴C] cholesterol and production of CE between C16:0 PS/C18:1 PC* and C18:1 PS/C16:0 PC*. The C18:1 chain in PC may have more impact on the synthesis of CE compared with the C18:1 in PS.

Second, enzymatic selectivity may also be involved in transport and metabolism. The C18:1* in C18:1 PS* liposomes was used to synthesize CE during lipid droplet formation in the process of forming foam cells. When cells are treated with C18:1 PS liposomes containing trace amounts of C16:0 PC*, which is enough to induce foam cell formation, the C16:0* fatty acids in C18:1 PS (C16:0*) liposomes could be released from PC, and converted to acyl-CoA, which is used to synthesize SM. Similar results were obtained from the experiments with pre-labeling of cellular fatty acids. When we examined the metabolism of cellular fatty acyl chains derived from phospholipids by pre-labeling the cells with [¹⁴C]C16:0 or [¹⁴C]C18:1 before adding C18:1 PS liposomes, the [¹⁴C]C18:1 was predominantly used to synthesize CEs and TG. However, [¹⁴C]C16:0 was used to synthesize SM. These differences in the metabolism of C16:0 and C18:1 could be caused by a fatty acid preference for enzymatic activity, which may involve the esterification of cholesterol to form CE by acyl-CoA cholesterol acyltransferase, as well as serine-palmitoyl transferases and ceramide syntheses. These results suggest that SM synthesis using C16:0 is accompanied by CE accumulation during foam cell formation. It has been established that accumulation of excess free cholesterol is toxic to cells.^{30,31)} Our results suggest that SM synthesized from C16:0 might attenuate the cytotoxicity caused by free cholesterol by forming lipid rafts in the solid membrane.

In the present study, we used a model system composed of PS liposomes and mouse peritoneal macrophages. Several studies have investigated the role of particle size in phagocytosis.^{32–35)} Tabata and Ikada studied the effect of the size of polymer microspheres on phagocytosis by mouse peritoneal macrophages, and reported that maximal phagocytosis was observed when the size was 1.0–2.0 μm.³⁵⁾ Champion *et al.* studied the phagocytosis of polymeric microspheres by rat alveolar macrophages, murine peritoneal macrophages, and human spleen macrophages.³⁶⁾ They concluded that particles in the range of 2–3 μm exhibited maximal phagocytosis. In our study, the sizes of the C16:0 PS liposomes and C18:1 PS liposomes were 1.26±0.0524 and 0.803±0.0838 μm, respectively (mean±standard deviation (S.D.), *n*=3). On the other hand, the sizes of C16:0 PC liposomes and C18:1 PC liposomes were much bigger (5.33±0.339 and 2.93±0.235 μm, respectively, mean±S.D., *n*=3) compared with the PS liposomes. Although the precise dependence of phagocytosis on the particle size remains unclear, we cannot rule out the possibility that the variation in the sizes of the liposomes, not only the charge of the PS, could have influenced the results. In addition, Kruth has claimed that macrophage metabolic pathways depend on

the origin of the cells used or species of lipids added.⁸⁾ Therefore, further study is needed to understand how closely our model system correlates with the actual situation *in vivo*.

Furthermore, liposomes have not only been a model for biological membranes but are also used as drug delivery systems, particularly in cancer treatment. Although a variety of phospholipid-related formulations have been found effective for the delivery of drugs in recent years, the currently available liposomal-based formulations do not exhibit sufficiently active targeting of cancer cells at a molecular level.^{37,38)} PS normally localizes to the inner leaflet of cell membranes but becomes exposed on the outer surface of tumor vascular endothelial cells^{39,40)} and also becomes exposed at the outer plasma membrane of apoptotic cells and activated platelets.⁴¹⁾ Therefore, it is worth considering the use of PS liposomes for not only the treatment of cancer but also atherosclerosis and inflammatory diseases, which are both associated with macrophages.

Taken together, the findings of the present study reveal that macrophages may have a fatty acid preference for the uptake of liposomes and the transport and esterification of cholesterol during foam cell formation.

Acknowledgments The authors would like to thank Drs. Kazuo Maruyama and Ryo Suzuki (Department of Biopharmaceutics, School of Pharmaceutical Sciences, Teikyo University) for their help on measuring the sizes of liposomes. Funding: This work was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI [Grant numbers 26461532 (K.Y.), 15K01691 (K.H.), 15K08625 (Y.F.)]. We thank Victoria Muir, Ph.D., from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

Conflict of Interest The authors declare no conflict of interest.

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