Increased lipid rafts and accelerated lipopolysaccharide-induced tumor necrosis factor-α secretion in Abca1-deficient macrophages

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Abstract  Lipid rafts on the cell surface are believed to be very important as platforms for various cellular functions. The aim of this study was to know whether defective lipid efflux may influence lipid rafts on the cell surface and their related cellular functions. We investigated macrophages with defective lipid efflux from ATP binding cassette transporter A1-deficient (Abca1-KO) mice. Lipid rafts were evaluated by following the two novel probes: a biotinylated and protease (subtilisin Carlsberg)- nicked derivative of α-toxin and a fluorescein ester of polyethylene glycol-derived cholesterol. Lipid rafts in Abca1-KO macrophages were increased, as demonstrated by both probes. Moreover, activities of nuclear factor κB, mRNA and intracellular distribution, and secretion of tumor necrosis factor-α (TNF-α) were examined after stimulation by lipopolysaccharides (LPSs). LPS-induced responses of the activation of nuclear factor κB and TNF-α were more prompt and accelerated in the Abca1-KO macrophages compared with wild-type macrophages. Modification of lipid rafts by cyclodextrin and nystatin corrected the abnormal response, suggesting an association between the increased lipid rafts and abnormal TNF-α secretion. We report here that Abca1-KO macrophages with defective lipid efflux exhibited increased lipid rafts on the cell surface and accelerated TNF-α secretion.—Koseki, M., K. Hirano, D. Masuda, C. Ikegami, M. Tanaka, A. Ota, J. C. Sandoval, Y. Nakagawa-Toyama, S. B. Sato, T. Kobayashi, Y. Shimada, Y. Ohno-Iwashita, F. Matsuura, I. Shimomura, and S. Yamashita. Increased lipid rafts and accelerated lipopolysaccharide-induced tumor necrosis factor-α secretion in Abca1-deficient macrophages. J. Lipid Res. 2007. 48: 299–306.

Supplementary key words  ATP binding cassette transporter A1 • biotinylated and protease (subtilisin Carlsberg)- nicked derivative of α-toxin • cholesterol efflux • lipid rafts • polyethylene glycol-derived cholesterol • tumor necrosis factor-α

Reverse cholesterol transport (RCT) is one of the major protective systems against atherosclerosis, in which HDL particles play a crucial role as a shuttle carrying cholesterol derived from peripheral tissues to the liver (1). Cholesterol efflux from the cells is the initial step of RCT, in which free apolipoprotein A-I (apoA-I) or small HDLs take up cholesterol from the peripheral cells. We have been trying to elucidate the molecular mechanism for RCT and cholesterol efflux by analyzing the pathophysiology of patients with abnormal HDL metabolism. We have identified molecules involved in cellular cholesterol efflux and apoA-I and HDL binding proteins on macrophages (2–5).

Tangier disease (TD) is a model for the impairment of cholesterol efflux from the cells (6, 7). Patients with TD suffer from genetic HDL deficiency, hepatosplenomegaly, orange tonsils, and premature atherosclerosis (8, 9). Many laboratories including ours have reported that mutations in the Abca1 gene lead to defective cholesterol efflux from the cells (10–12). As a result of the mutation(s) in the Abca1 gene, cells from TD patients exhibited a deficiency of apoA-I-mediated cholesterol efflux and a subsequent accumulation of intracellular lipids as lipid droplets, which is closely related to the development of atherosclerosis in this disorder.

On the other hand, in the plasma membrane, cholesterol is distributed abundantly in some domains structures

Abbreviations: Abca1-KO, ATP binding cassette transporter A1-deficient; apoA-I, apolipoprotein A-I; BCR, biotinylated and protease (subtilisin Carlsberg)- nicked derivative of α-toxin; fPEG-chol, fluorescein polyethylene glycol cholesterol ether; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; 2OHpβCD, 2-hydroxypropyl-β-cyclodextrin; RCT, reverse cholesterol transport; TD, Tangier disease; TNF-α, tumor necrosis factor-α; WT, wild-type.

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called “lipid rafts,” “cholesterol-rich microdomains,” or “detergent membranes” (13). These domains are enriched in cholesterol and sphingolipids and contain specific proteins, including glycosylphosphatidylinositol-anchored proteins, and are believed to be important as rafts mediating some intracellular and/or extracellular signals (14–20). Recently, the following two probes were developed to visualize rafts. One is a biotinylated and protease (subtilisin Carlsberg)-nicked derivative of ß-toxin (BCO) (21–25). This probe was developed by Ohno-Iwashita et al. (21–25) and is derived from a thiol-activated cytolysin produced by Clostridium perfringens. BCO selectively binds to membrane cholesterol in lipid rafts. The other probe is a polyethylene glycol cholesterol ether (26, 27). This compound belongs to a unique group of nonionic amphipathic cholesterol derivatives. It can bind with cholesterol-rich membranes both in cells and in model membranes. It was recently reported that a fluorescent polyethylene glycol cholesterol ether (fPEG-chol) is a sensitive probe for unraveling the dynamics of cholesterol-rich microdomains in living cells.

Little is known about the effect of defective lipid efflux on lipid rafts in plasma membranes. In this study, we have tested the hypothesis that defective efflux influences lipid rafts in the plasma membrane and examined related cellular functions using ATP binding cassette transporter A1-deficient (Abca1-KO) macrophages as a model.

METHODS

Materials

BCO and fPEG-chol were prepared as described previously (26, 28).

Animal treatment and cell culture

Abca1-KO mice created on the DBA1 lac/J background (29) were purchased from the Jackson Laboratory. Mice were fed a normal chow diet. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine. After an intraperitoneal injection of 2 ml of 4% thioglycollate (B-255; Sigma-Aldrich) medium, mouse peritoneal macrophages were harvested from Abca1-KO and wild-type (WT) mice. The cells were cultured according to standard conditions in Dulbecco’s minimum essential medium supplemented with L-glutamine, nonessential amino acids, and 10% fetal calf serum in a humidified 5% CO2 atmosphere at 37°C. Human monocyte-derived macrophages were obtained from healthy volunteers. The cells were cultured according to the protocol of the manufacturer’s protocols (TransAM NF-κB p65 Chemi; Active Motif). Nucleoproteins were extracted from the macrophages (Nuclear Extract Kit; Active Motif), and nuclear factor-κB (NF-κB) p65 activity was measured according to the manufacturer’s protocols (TransAM NF-κB p65 Chemi; Active Motif).

RNA isolation, cDNA synthesis, and quantitative PCR

Total RNA was isolated from mouse macrophages using the RNasy Mini Kit (Qiagen), followed by treatment with DNase I (Qiagen). Of each RNA sample, 1 μg of total RNA was primed with 50 pmol of oligo(dT)20 and reverse-transcribed with SuperScript III RT (200 units; Invitrogen), according to the protocol of the manufacturer. Real-time quantitative PCR was performed according to the protocol of the DyNAmo HS SYBR Green quantitative PCR kit. To assess the expression levels of TNF-α mRNA in macrophages, DNA polymerase and SYBR Green I were used in a reaction volume of 20 μl using gene-specific primers (5 mM) on cDNA (corresponding to ~50 ng of total RNA) (8, 30).

Primers used in this study

Each set of primers located different exons: primer 1, 5’-cagcacccctacactcagatca-3’; mouse TNF-α cDNA, nucleotides 381–402 (GenBank accession number NM_013693); primer 2, 5’-cctggttctgctagctacg-3’; mouse TNF-α cDNA, nucleotides 459–439 (GenBank accession number NM_013693); primer 3, 5’-ggagcagccagggcagatacct-3’; mouse GAPDH cDNA, nucleotides 383–405 (GenBank accession number M32599); primer 4 was designed for each fluorescent probe by confocal laser microscopy (LSM510; Carl Zeiss).

Fractionation by sucrose density gradient ultracentrifugation

After incubation with 10 μg/ml BCO for 30 min on ice, mouse peritoneal macrophages were harvested and sucrose gradient ultracentrifugation was performed as described previously (25). Free cholesterol levels were measured using the Amplex Red cholesterol assay method (Molecular Probes) (25).

Detection of BCO bound to cells by Western blot analysis

The lysates of BCO-bound cells were subjected to SDS-PAGE, and Western blot analysis was performed as described previously (25).

Construction of adenovirus vectors and their expression in fibroblasts

FLAG-tagged human Abca1 cDNA with the FLAG epitope (DYKDDDK) incorporated at its C terminus (hAbca1/FLAG) was generated by PCR. Adenovirus vectors encoding LacZ and hAbca1/FLAG were constructed according to the protocol of the Adeno X expression system (Clontech). Infection with adenovirus was carried out by incubating cells in serum-free medium for 1 h at 37 °C under gentle agitation. After incubation, complete medium was supplied, and the cells were further incubated in a CO2 incubator. Five days after infection with the indicated multiplicity of infection, the cells were used in the experiments.
RESULTS

Lipid rafts were increased in Abca1-KO macrophages

Lipid rafts on the plasma membrane were visualized using two different probes, BC0 and fPEG-chol. Confocal laser scanning microscopy revealed that both probes recognized a greater volume of lipid rafts in the mouse peritoneal macrophages from Abca1-KO mice than from WT mice (Fig. 1A). Similar results were obtained in human monocyte-derived macrophages from a TD patient (9) and from normal subjects (Fig. 1B). These results suggested that lipid rafts on the plasma membrane were increased in Abca1-KO macrophages. As the number of the patient macrophages was limited, we made use of mouse macrophages in the experiments described below.

To confirm whether BC0 recognizes cholesterol-rich domains in mouse peritoneal macrophages, sucrose density gradient ultracentrifugation was performed. In both Abca1-KO and WT macrophages, the distribution of free cholesterol concentration consisted of two peaks: low density raft fractions (fractions 2–5) and high density nonraft fractions (fractions 8–10) (Fig. 2A). The sums of cholesterol content in the raft fractions (fractions 2–5) and in the nonraft fractions (fractions 8–10) were calculated, showing that the free cholesterol content of lipid rafts was increased significantly in Abca1-KO macrophages and that the free cholesterol content of nonrafts was not significantly different between WT and Abca1-KO macrophages (Fig. 2B). As expected, BC0 was distributed mainly in low-density, Triton X-100-insoluble membrane fractions (fractions 2–5), as shown by Western blot analysis, in both Abca1-KO and WT macrophages, and BC0 binding to rafts of Abca1-KO macrophages was detected more strongly than to rafts of WT macrophages (Fig. 2A).

Abca1 complementation corrected abnormal lipid rafts

Furthermore, to analyze the relationship between Abca1 and lipid rafts, we performed a complementary experiment using TD fibroblasts. The lipid rafts in TD fibroblasts were increased (see supplementary Fig. IA). We generated the adenovirus encoding human Abca1 and examined its effect on binding of BC0 to TD fibroblasts. Confocal laser scanning microscopy and Western blot analysis clearly showed a multiplicity of infection-dependent decrease in BC0 binding (see supplementary Fig. IB). This result indicated that introduction of the Abca1 gene corrected the phenotype of TD fibroblasts, suggesting a causal relationship between Abca1 deficiency and the alteration of lipid rafts.

Abnormal cytokine secretion from Abca1-KO macrophages

We supposed that the increase of lipid rafts, as well as intracellular lipid storage, might affect the process of premature atherosclerosis in patients with TD. We hypothesized that the increase of lipid rafts might affect the activation of nuclear receptors and the subsequent synthesis and secretion of cytokines in macrophages, because some papers reported that lipid rafts might play a pivotal role in the cellular recognition of LPS (31). Therefore, we focused on LPS-induced intracellular signaling and cytokine secretion, particularly at an acute phase after...
LPS stimulation. As shown in Fig. 3A, activities of NF-κB p65 were induced only at 15 min in both Abca1-KO and WT macrophages, and the activity was significantly higher in Abca1-KO macrophages than in WT macrophages throughout the time course. Figure 3B shows the TNF-α mRNA levels. TNF-α mRNA levels were induced at 30 min and reached a peak at 45 min in both Abca1-KO and WT macrophages. TNF-α mRNA in Abca1-KO macrophages was significantly higher than in WT macrophages at any time point after 30 min. Figure 3C demonstrates the immunocytochemical analysis for TNF-α (32). Before LPS stimulation, we could not detect the immunoreactive mass of TNF-α in either macrophages. After 1 h, the immunoreactive mass of TNF-α was detected in perinuclear organelles in Abca1-KO macrophages but not in WT macrophages. After 2 h, the immunoreactive mass of TNF-α was found more dispersed in the cytoplasm of Abca1-KO macrophages. Figure 3D shows TNF-α secretion data. A significant difference in TNF-α secretion into the medium occurred at 1 h after LPS stimulation. Similarly, the secretion of interleukin-6 into the medium was significantly higher in Abca1-KO macrophages than in WT macrophages (see supplementary Fig. II). These data suggest that an acute phase response to LPS seems to be accelerated in Abca1-KO macrophages.

Effect of lipid raft modulators on mRNA and release of TNF-α

Finally, we investigated the relationship between increased lipid rafts and the accelerated response of TNF-α in Abca1-KO macrophages. We used the following two lipid raft modulators: 2-hydroxypropyl-β-cyclodextrin (2OHpβCD) and nystatin (21, 31, 33). Figure 4A shows the effect of 2OHpβCD, which selectively depleted cholesterol from lipid rafts, on staining with BCθ. The signals of BCθ in the bottom fraction 11 seemed to be free BCθ. The results shown are representative of three independent experiments. B: The sums of cholesterol contents in lipid raft fractions (fractions 2–5) and in nonlipid raft fractions (fractions 8–10) were compared between Abca1-KO and WT macrophages. Free cholesterol contents were significantly higher in lipid rafts of Abca1-KO macrophages than in those of WT macrophages. Values shown are mean ± SEM. * P < 0.05 by Student’s t-test.
KO and WT macrophages. Next, as shown in Fig. 4C, we tested another lipid raft modulator, nystatin, which was shown to disrupt cholesterol-rich domains. After treatment with nystatin, the expression of TNF-α was also decreased significantly in both macrophages. Treatment with 25 μg/ml nystatin attenuated the significant difference of TNF-α mRNA levels between Abca1-KO and WT macrophages. These data strongly suggested that Abca1-KO macrophages were more affected by lipid raft modulators because of the increase of lipid rafts. The alteration of lipid rafts may regulate the acute response of TNF-α by LPS stimulation.

DISCUSSION

Lipid rafts and atherosclerosis

Lipid bilayers in the plasma membrane were previously believed to be homogeneous. Recently, a number of studies revealed that lipid rafts could play an essential role in many cellular processes, including signal transduction, membrane trafficking, cytoskeletal organization, and many other cellular events (17–20, 34, 35). Even though many studies focused on the distribution pattern of membrane proteins in lipid rafts, there is little evidence that a particular genetic defect might affect the number of lipid rafts and subsequent cellular functions. One reason for this might be the complexity and difficulty of measuring lipid rafts. In this study, we have succeeded relatively easily in comparing the volume of lipid rafts using two newly developed lipid raft probes. Here, for the first time, we report that a mutation in a single gene might alter lipid rafts and that the increase in lipid rafts might be related to the acceleration of atherogenic processes. In this study, we focused on the acute secretion to LPS stimulation. Recently, Ishiwata et al. (26) and Kay et al. (36) reported on the importance of cholesterol-rich lipid rafts in the delivery of TNF-α to the plasma membrane and the exit sites for cytokine secretion. It would be of interest to investigate whether the increased lipid rafts in the Abca1-KO macrophages might affect the exocytosis of cytokines. Further studies will be necessary to clarify this.
Abca1 deficiency may accelerate atherosclerosis mediated by the increase of lipid rafts

TD is a familial HDL deficiency, which is a model for impaired cholesterol efflux, the initial step of RCT, and is frequently associated with cardiovascular diseases. We previously reported that a patient with TD suffered from severe coronary atherosclerosis using intravascular ultrasoundography (9). Many previous studies have indicated that TD is associated with mutations in the Abca1 gene and that the loss of function of Abca1 led to defective cholesterol and phospholipid efflux from macrophages, followed by intracellular lipid accumulation, foam cell formation, and atherosclerosis (Fig. 5, left). In this study, we have shown another aspect of TD: that impaired cholesterol efflux may cause the deposition of free cholesterol in lipid rafts (Fig. 5, right) and that the increased lipid rafts may play a principal role in the regulation of the acute response of TNF-α to LPS stimulation. These observations may indicate that premature atherosclerosis in patients with TD may be accelerated by enhanced inflammation through an abnormality of lipid rafts. In vivo, the increased TNF-α levels in the plasma of Abca1-KO mice or TD patients have not been reported. Further studies will be necessary to clarify this issue.

During the preparation of our manuscript, Landry et al. (37) reported the unique relationship between Abca1 expression and membrane microdomains. They used BHK cells expressing a functional Abca1 or a nonfunctional Abca1 with a mutation in the ATP binding domain. They clearly showed that the overexpression of Abca1 results in the disruption of microdomains through its ATPase-related functions. On the other hand, we focused on the cause of TD and independently investigated the macrophages derived from a patient with TD and Abca1-KO mice. We demonstrated the increase in lipid rafts with two newly developed probes recognizing cholesterol directly
and suggested accelerated TNF-α secretion under Abca1-KO conditions. Together, these data strongly demonstrate that Abca1 might be involved in the regulation and formation of lipid rafts in plasma membranes.

**Cholesterol deposition and inflammation in macrophages**

It is widely believed that inflammation might contribute to the progression of atherosclerosis. However, the relationship between intracellular lipid storage and inflammation in macrophages has not been clarified. Some studies revealed the relationship between cholesterol deposition and cytokine secretion in macrophages. Li et al. (38) demonstrated that TNF-α and interleukin-6 were induced in free cholesterol-loaded macrophages without LPS stimulation. They speculated that an excess storage of endoplasmic reticulum cholesterol may be the cause. Our data raised the possibility that cholesterol deposition in plasma membranes might affect the accelerated induction of TNF-α. On the other hand, Francone et al. (39) reported an observational study about the intracellular lipid storage and proinflammatory conditions of Abca1 and LDL receptor double knockout macrophages. In our study, we revealed one of the molecular mechanisms for this, showing that the increased lipid rafts may result in abnormal cytokine release.

**Lipid rafts as a therapeutic target**

Abca1 plays a key role in the regulation of cholesterol homeostasis and the function of macrophages. We demonstrated the relationship between Abca1 and lipid rafts using Abca1-defective animal and human models. On the other hand, in Abca1-expressing macrophages, the function of Abca1 was altered under some conditions. Wang and Oram (40, 41) reported that unsaturated fatty acids reduced Abca1 expression in macrophages by enhancing its degradation rate. These findings might support the speculation that the Abca1 of normal macrophages might be destabilized by unsaturated fatty acids, resulting in alterations of lipid rafts. The alteration of lipid rafts should be investigated in other atherogenic conditions affecting lipid efflux. In this study, we demonstrated that an extremely short-time modification of lipid composition in plasma membranes, using lipid raft modulators, might be a novel therapeutic strategy to attenuate acute accelerated proinflammatory events in macrophages.

**Conclusion**

Abca1-KO macrophages with defective lipid efflux exhibited increased lipid rafts on the cell surface and accelerated TNF-α release, which is a novel phenotype of macrophages with defective lipid efflux. Thus, modulation of Abca1 and lipid rafts may become a novel therapeutic target to prevent atherosclerosis.

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