Visualizing caveolin-1 and HDL in cholesterol-loaded aortic endothelial cells

W. T. Chao,* S. S. Fan,* J. K. Chen,† and V. C. Yang[1],*

Department of Biology and Life Science Research Center,* Tunghai University, Taichung, Taiwan, Republic of China; and Department of Physiology,† College of Medicine, Chang Gung University, Taoyuan, Taiwan, Republic of China

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Abstract Caveolae are vesicular invaginations of the plasma membranes that regulate signal transduction and transcytosis, as well as cellular cholesterol homeostasis. Our previous studies indicated that the removal of cholesterol from aortic endothelial cells and smooth muscle cells in the presence of HDL is associated with plasmalemmal invaginations and plasmalemmal vesicles. The goal of the present study was to investigate the location and distribution of caveolin-1, the main structural protein component of caveolae, in cholesterol-loaded aortic endothelial cells after HDL incubation. Confocal microscopic analysis demonstrated that the caveolin-1 appeared to colocalize with HDL-fluorescein 1,1′-dioctadecyl 3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) conjugates on the cell surface. No free HDL-DiI conjugates were revealed in the cytoplasm. Immunoelectron microscopy further demonstrated that caveolin-1 gold (15 nm) conjugates colocalized with HDL gold (10 nm) conjugates in the plasmalemmal invaginations. These morphological results indicated that caveolae are the major membrane domains facilitating the transport of excess cholesterol to HDL on the cell surface of aortic endothelial cells.—Chao, W. T., S. S. Fan, J. K. Chen, and V. C. Yang. **Visualizing caveolin-1 and HDL in cholesterol-loaded aortic endothelial cells.** ***J. Lipid Res.*** **2003.** 44: **1094–1099.**

Supplementary key words high density lipoprotein • colocalization • atherosclerosis

It has been demonstrated that HDL mediates the transport of cholesterol from peripheral tissues to the liver through a reverse cholesterol transport pathway (1–3); however, there is still considerable debate about the cellular mechanism by which HDL removes excess cholesterol from cells. One current theory holds that cholesterol efflux from cells to HDL involves an endocytotic pathway and apolipoprotein particles resecreted by retroendocytosis in intestinal epithelial cells, hepatocytes, and macrophages (4–7). Another theory supports the concept that HDL docks to a cell surface receptor, which triggers a signal that leads to the delivery of cholesterol to HDL without the necessity for particle uptake (8–10). Thus, the underlying mechanism of HDL-mediated cholesterol efflux is not fully resolved.

The noncoated vesicular structures "caveolae," which have been implicated in transmembnrane transport (11), were recognized as flask-shaped indentations of the cell membrane a half century ago (12). Recently, several reports have indicated that these membrane domains appear to play several important cellular roles in targeting of various molecules involved in signal transduction (13). These membrane domains contain caveolin-1, a main structural component of caveolae, and are particularly rich in cholesterol and sphingolipids (14). This unique structure has led to the hypothesis that they may play an important role in the regulation of cellular cholesterol homeostasis (15). Biochemical study has demonstrated that chronic HDL exposure can down-regulate caveolin-1 expression in cholesterol-loaded NIH/3T3 cells (16). Caveolin-1 is involved in enrichment with cholesterol of HDL generated by the apolipoprotein-cell interaction in THP-1 cells and promotes cellular cholesterol release (17). Cells in caveolin-1 knockout mice show no signs of caveolae structure (18). Depletion of cholesterol and caveolin-1 results in a noninvaginated morphology on the cell surface (19, 20). Our previous studies indicated that the removal of excess cholesterol from aortic endothelial cells and smooth muscle cells in the presence of HDL is facilitated by the plasmalemmal invaginations and plasmalemmal vesicles (21); however, the interaction between caveolin-1 and HDL at the substructural level in cholesterol-loaded endothelial cells is still unknown. This information is important to further elucidate the underlying mechanisms of HDL-mediated cholesterol efflux from the cell. In this study, we used confocal microscopy and immunoelectron

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[1]To whom correspondence should be addressed.
e-mail: vcyang@mail.thu.edu.tw

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microscopy to investigate the location and distribution of caveolin-1 in cholesterol-loaded aortic endothelial cells after HDL exposure. Our results indicated that HDL was colocalized with the caveolin-1 in the plasmalemmal invaginations.

MATERIALS AND METHODS

Cell culture

Sprague-Dawley rats 4 weeks old were sacrificed. Rings 1 mm thick were cut from the thoracic aorta, cultured at 37°C in Dulbecco's minimum essential medium (DMEM), and supplemented with 10% FBS and 50 U/ml penicillin-streptomycin in an 5% CO_2-95% air atmosphere. In order to establish pure endothelial cell cultures, ring explants were removed after 3 to 4 days of culture (22). Cultures exhibiting pure endothelial cells, maintained for 2–7 passages, were used in the experiment.

Prior to the experiment, subconfluent monolayers of endothelial cells were washed twice with PBS containing 2 mg/ml fatty acid-free albumin (FAFA) and incubated with DMEM containing 2 mg/ml FAFA and 50 μg/ml cholesterol in ethanol (10 mg/ml) for 48 h at 37°C.

Localization of HDL and caveolin-1 by confocal microscopy

Cholesterol-loaded cells grown on a cover slip in a 24-well plate for 48 h were washed three times with PBS-albumin followed by incubation with DMEM containing 50 μg/ml HDL-fluorescein 1,1′-dioctadecyl 3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and 2 mg/ml FAFA at 37°C for 5 min. The cells were then chilled on ice, washed three times with ice-cold PBS-albumin for 15 min, fixed in cold methanol for 5 min, and air dried. After washing with PBS, cover slip cultures were incubated with rabbit anti-human caveolin-1 antibody (1:200, v/v) (Santa Cruz, CA) or monoclonal anti-β-actin antibody (1:1,000, v/v) (Sigma) for 30 min. Cover slips were then washed three times with PBS and incubated for 30 min with FITC-conjugated goat anti-rabbit IgG (1:300, v/v) followed by Cy_5-conjugated rabbit anti-mouse IgG (1:300, v/v). Cover slips were washed three times in PBS, mounted on slides, and photographed with an LSM 510 confocal microscope (Zeiss, Germany). A 3-D view was composed from Z-section scanning images and analyzed by the LSM 510 image system (Zeiss, Germany).

HDL-colloidal gold conjugates

Colloidal gold, 6–10 nm in diameter, was prepared by heating 50 ml of 0.01% $HAuCl_4$ to 60°C then adding 0.9 ml of 1% trisodium citrate $2H_2O$ prewarmed to 60°C. The mixture was heated to boiling for 5 min to obtain a clear red solution. To 5 ml of the colloidal gold solution, 0.5 ml human HDL (2 mg ml) in PBS with 0.05 M EDTA at pH 5.5 was added rapidly, and the mixture was manually shaken. To remove the excess free HDL, the conjugates were centrifuged at 9,000 *g* on a 70 Ti rotor (Beckman L80) for 30 min. The pellet was resuspended in 1 ml PBS, pH 7.4, by sonication. Before use, the conjugates were filtered through a 0.22 μm Millipore filter (23, 24).

Immunoelectron microscopy

The cholesterol-loaded cells were washed three times with PBS-albumin and incubated with DMEM and 2 mg/ml FAFA containing 80 μg/ml HDL gold (diameter = 10 nm) conjugates at 37°C for 5 min. After incubation, the cells were chilled on ice, washed three times with ice-cold PBS-albumin, then prefixed with 4% paraformaldehyde in PBS for 30 min. After washing, the prefixed

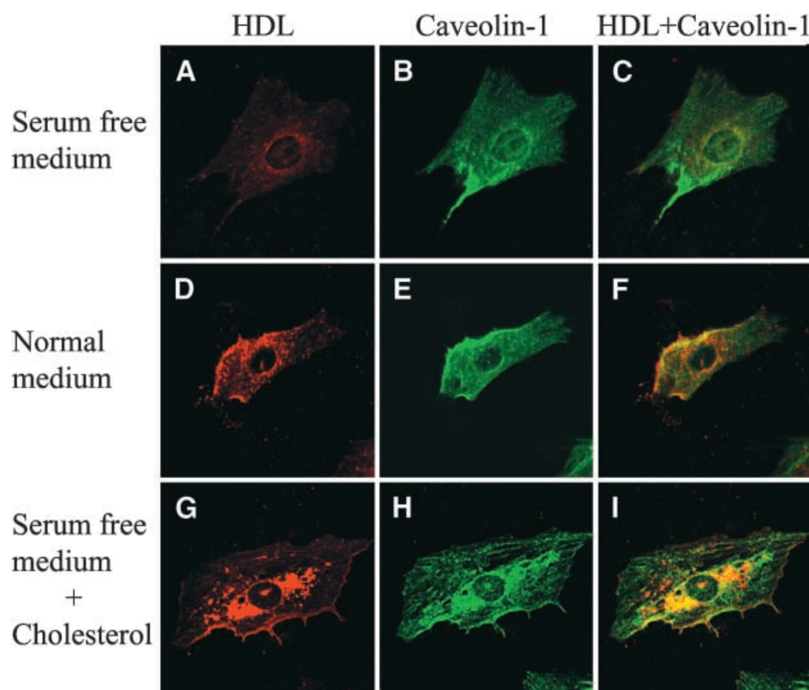

Fig. 1. The confocal micrographs showed that when cells were incubated in serum-free medium for 24 h followed by HDL incubation for 5 min, the cells showed a very low level of fluorescence (red, HDL; green, caveolin-1) throughout the cytoplasm and on the cell surface (A–C). When the cells were incubated in normal medium followed by HDL incubation, cells showed more fluorescence (D–F). When the cells were incubated in the cholesterol medium for 48 h, the intensity of fluorescence increased (G, H). The HDL colocalized with caveolin-1 (I).

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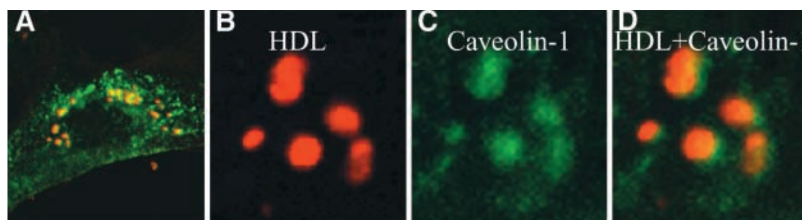

Fig. 2. A cholesterol-loaded endothelial cell showing the colocalization of HDL (red fluorescence) and caveolin-1 (green fluorescence) (A). Enlarged images of A show the colocalization of HDL and caveolin-1 (B–D).

cells were incubated with rabbit anti-caveolin-1 primary antibody (1:1,000 in PBS-albumin) for 1 h. The cells were then washed four times with PBS-albumin and incubated with goat anti-rabbit IgG conjugated with 15 nm colloidal gold (1:40 in PBS-albumin) for 1 h. After washing four times with PBS-albumin, the cells were postfixed with 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C for 2 h (Lin et al.). The cells were then incubated with 1% OsO_4 for 1.5 h at 4°C, dehydrated in a graded ethanol series, and embedded in LX112 (EMS). Sections of 0.6 μm thickness were stained with toluidine blue for light microscopy. Thin sections (80 nm) were stained with uranyl acetate and lead citrate and viewed under a Hitachi H-600 transmission electron microscope (Hitachi, Japan).

RESULTS

Localization of HDL and caveolin-1 in cholesterol-loaded endothelial cells

Immunofluorescence confocal microscopy. The localization of caveolin-1 and HDL in the cholesterol-loaded endothelial cells was performed by immunofluorescence confocal microscopy. The results showed that when the endothelial cells were incubated in serum-free medium for 24 h, only weak signals of caveolin-1 and HDL-(DiI) were revealed in the cell (**Fig. 1A–C**). When the cells were incubated in the normal culture medium without cholesterol (Fig. 1D–F), the signals of HDL-DiI and caveolin-1 increased (Fig. 1D). When cells were incubated in the culture medium with cholesterol for 48 h (Fig. 1G–I), large amounts of HDL-DiI appeared around the nuclear region (Fig. 1G). Furthermore, the HDL-DiI and caveolin-1 were revealed in a similar distribution pattern (Fig. 1I) and colocalized (**Figs. 2**, **3A–C**). The 3-D confocal image of a top view showed that the HDL-DiI colocalized with caveolin-1 predominantly on the cell surface (Fig. 3D). In a side view of an endothelial cell, β-actin was present in the cytoplasm and caveolin-1 was on the cell surface (**Fig. 4A–C**). The HDL-DiI was revealed on the cell surface (Fig. 4B) or invaginations into the cell (Fig. 4C). These results demonstrate that the HDL was located only on the cell membrane or on invaginated membrane structures (Fig. 4D).

Immunoelectron microscopy. The localization of HDL and caveolin-1 was also observed by immunoelectron microscopy. The cholesterol-loaded endothelial cells were first incubated with HDL gold (10 nm in diameter) complexes at 37°C for 5 min. After prefixation, the cells were incubated with rabbit anti-human caveolin-1 antibody followed by colloidal gold (15 nm in diameter)-conjugated goat anti-rabbit secondary antibody. The caveolin-1 colloidal

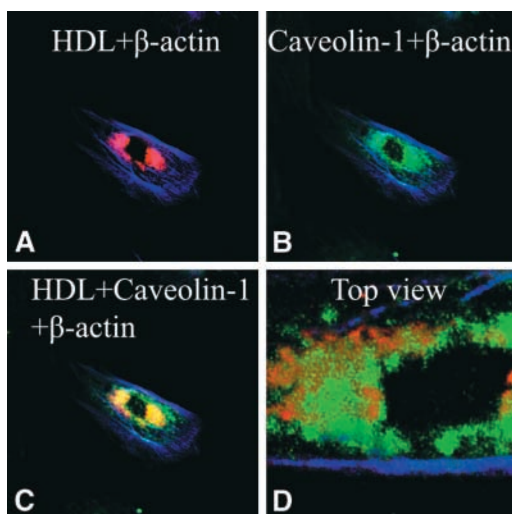

Fig. 3. The 3D confocal images of an endothelial cell stained with HDL, caveolin-1, and β-actin. The green fluorescence and red fluorescence indicate the locations of caveolin-1 and HDL, respectively (A–C), and the blue color represents β-actin. A top view of the image shows the HDL colocalized with caveolin-1 on the cell membrane (D).

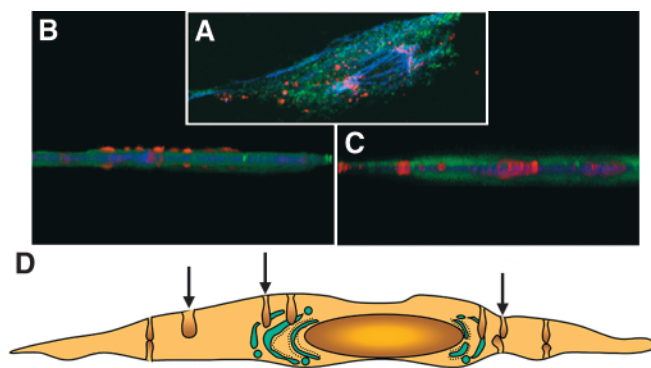

Fig. 4. The 3D confocal images of another endothelial cell stained with HDL (red fluorescence), caveolin-1 (green fluorescence), and β-actin (blue fluorescence) (A–C). In a lateral view of the image (B, C), β-actin was revealed in the cytoplasm, and caveolin-1 (green) was distributed on the cell surface. HDL (red) was only revealed on the cell surface or in invaginations through the cytoplasm (B, C). D: A cartoon diagram showing the HDL located on the cell membrane invaginations (arrow).

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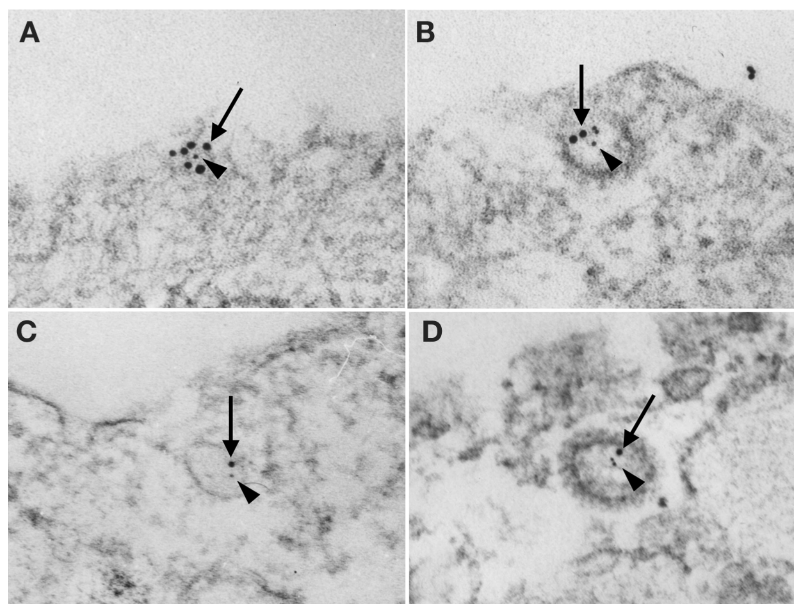

Fig. 5. The immunoelectron micrographs showed the colocalization of HDL (arrow head) and caveolin-1 (arrow) in the plasmalemmal invagination (A, B) and plasmalemmal vesicle (C). Only a few HDL gold and caveolin-1 gold conjugates were found in the coated vesicle (D).

gold particles were observed in the plasmalemmal invaginations (**Fig. 5A**, **B**) and plasmalemmal vesicles (Fig. 5C). In addition, the caveolin-1 colocalized with HDL in the plasmalemmal invaginations (Fig. 5A, B) as well as in the plasmalemmal vesicles (Fig. 5C). Occasionally, the colocalization of caveolin-1 and HDL was observed in coated vesicles (Fig. 5D). A few free HDL gold complexes were observed in the cytoplasm.

DISCUSSION

The important role played by HDL in the removal of excess cholesterol from cells is well recognized; however, there is considerable debate on the mechanism involved in HDL-mediated cholesterol efflux from cells. Biochemical studies have identified specialized plasma membrane domains, "caveolae," as a major intermediate for the efflux of free cholesterol from cultured skin fibroblasts to HDL (1). Frank et al. demonstrated that chronic HDL-exposure reduced caveolin-1 expression in NIH/3T3 cells (16). A role of caveolin-1 in cholesterol release has been described in incorporation of cholesterol into the HDL generated by the apoA-I and cell interaction in THP-1 cells (17, 25). It has also been shown that caveolin-1 is translocated into cytosol fraction by apoA-1 stimulation in rat astrocyte (26, 27). This paper reports findings concerning the morphological aspects of the relationship between caveolin-1 and HDL in cholesterol-loaded aortic endothelial cells.

HDL is the main acceptor for cholesterol efflux from the cells. It has been suggested that HDL docking to specific cell surface receptors stimulates translocation of cholesterol from the intracellular cholesterol pool to the cell membrane through activation of phospholipases and protein kinase C (28, 29). Alternatively, a pathway of endosomal internalization and subsequent retroendocytosis of the HDL particle after enrichment with cholesterol has been suggested in rat hepatoma cells and macrophages by several investigators (5, 30); however, direct visualization of the distribution of HDL in cholesterol-loaded cells has been lacking. The vesicular structure of the endothelial cells and their role in vesicular transport have been questioned for many years (31). People argue that at least some of the vesicles revealed under the electron microscope appeared to be surface membrane invaginations instead of free vesicles; however, due to the limitation of microscopic techniques, there was no consensus until the invention of confocal microscopy in recent years. The three-dimensional structure of organelles and the distribution of fluorescent-labeled molecules or tissues can be investigated accurately under the confocal microscope. A previous study from our laboratory demonstrated that HDL-mediated cholesterol efflux in aortic endothelial cells and smooth muscle cells was closely related to plasmalemmal invaginations and plasmalemmal vesicles located near the cell surface (21). In this study, the confocal microscopic observations showed that most of the HDL colocalized with caveolin-1 on the cell surface (Fig. 3A). The caveolin-1 signals were revealed on the cell surface as well as in the cytoplasm; however, only a few signals of HDL were observed in the cytoplasm. Our electron microscopic results further demonstrated caveolin-1 in the plasmalemmal invaginations and plasmalemmal vesicles colocalized with HDL in cholesterol-loaded cells. Only a few free HDLs and caveolae were revealed in the cytoplasm. We therefore assume that most of the plasmalemmal vesicles near the cell surface revealed under the

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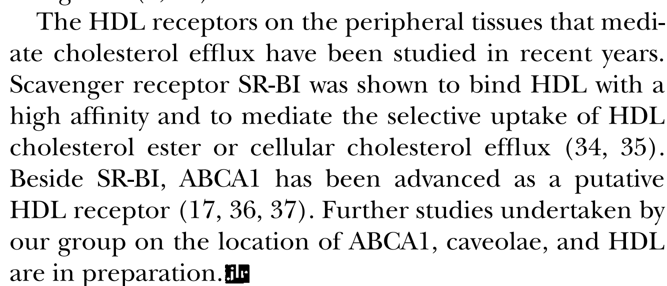
electron microscope are membrane invaginations instead of free vesicles. We propose that HDL might be docking to the specific membrane domain, caveolae, and stimulating the cholesterol efflux. Arakawa et al. have demonstrated that caveolin-1 is involved in enrichment with cholesterol of the HDL generated by the apolipoprotein-cell interaction (17). Further study needs to be done in order to elaborate this physiological significance. In the trypsin digestion experiment, HDL was present in the cell lysates of cholesterol-loaded cells without trypsin digestion, but when these cells were incubated at 4°C or at 37°C followed by trypsin digestion, HDL was not detected in the cell lysates (unpublished observations). This result might suggest that instead of entering the cell, most of the HDL is docking to the cell surface and translocating the free cholesterol from the cell to the cell surface by a signal transduction pathway, as proposed by several investigators (8, 28, 29).

Fielding's model demonstrated that the caveolae play an important role in protecting the cells against free cholesterol accumulation (32). An increase in cellular cholesterol up-regulated caveolin expression, which led to depletion of caveolar cholesterol by HDL, which in turn resulted in down-regulation of cell surface caveolin levels (32). In a separate experiment, we found that HDL exposure reduced caveolin-1 protein expression in the aortic endothelial cells (unpublished observations).

Our previous study found that when the cholesterol-loaded cells were incubated with HDL at 4°C, the cell membrane was smooth without protuberances. When the cells returned to 37°C for 5 min, plasmalemmal invaginations appeared (21). After 30 min of incubation with HDL, the cell membrane flattened out again and the plasmalemmal invaginations disappeared. The present study was the first ultrastructural study to demonstrate the relationship between caveolae and HDL in the HDL-mediated cholesterol efflux experiments performed by several investigators (8, 33).



The HDL receptors on the peripheral tissues that mediate cholesterol efflux have been studied in recent years. Scavenger receptor SR-BI was shown to bind HDL with a high affinity and to mediate the selective uptake of HDL cholesterol ester or cellular cholesterol efflux (34, 35). Beside SR-BI, ABCA1 has been advanced as a putative HDL receptor (17, 36, 37). Further studies undertaken by our group on the location of ABCA1, caveolae, and HDL are in preparation.

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