

## Cellular localization and interaction of ABCA1 and caveolin-1 in aortic endothelial cells after HDL incubation

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Received 29 April 2005

Available online 12 May 2005

### Abstract

The goal of this study was to investigate the cellular localization and the interaction between caveolin-1 and ABCA1 in cholesterol-loaded aortic endothelial cells after HDL incubation. Immunofluorescence confocal microscopy showed that ABCA1 was found primarily on the cell surface, whereas caveolin-1 was revealed on the cell surface and in the cytoplasm. The HDL appeared to colocalize with ABCA1 and caveolin-1 on the cell surface. No free HDL was revealed in the cytoplasm. The HDL was colocalized neither with early endosome marker (CD71) nor with late endosome marker (LAMP2). The chemical cross-linking and immunoprecipitation analysis revealed that ABCA1 binds directly to both HDL and caveolin-1, whereas HDL does not bind directly to caveolin-1. The studies provide evidence for a direct interaction between ABCA1 and HDL, ABCA1 and caveolin-1, but not HDL and caveolin-1, indicating that ABCA1 may act as a structural platform between HDL and caveolin-1 on the cell surface during cellular cholesterol efflux.

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**Keywords:** ABCA1; Caveolin-1; HDL; Colocalization

It has been demonstrated that high-density lipoprotein (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 and the underlying mechanism is not fully resolved [4–9]. Caveolae (i.e., plasmalemmal vesicles) are free cholesterol-rich, invaginated microdomains (60–80 nm in diameter) at the surface of most peripheral cells. They have been implicated in many cellular activities including transcytosis, potocytosis, and signal transduction [10–12]. Caveolin-1, a main structural component of caveo-

lae, has been implicated in the regulation of cellular cholesterol metabolism and lipid uptake, as well as efflux [13–16]. It has been demonstrated that 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]. Apolipoprotein A-I (apo A-I) was reportedly involved in cholesterol efflux mediated by HDL in the endothelial cells [18]. Over-expression of caveolin-1 in hepatic cells stimulates cholesterol efflux by enhancing transfer of cholesterol to cholesterol-rich domains of the plasma membrane [19]. Our morphological studies indicated that the removal of excess cholesterol from aortic endothelial cells and smooth muscle cells in the presence of HDL is facilitated by plasmalemmal invaginations and plasmalemmal vesicles [20]. We further demonstrated that HDL colocalized with caveolin-1 in these membrane invaginated structures in cholesterol-loaded

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endothelial cells [21]. However, the mechanism of interaction between HDL and caveolin-1 on the cell surface is still unknown.

A breakthrough in our understanding of transfer of cholesterol from peripheral cells to HDL came recently from studies of Tangier disease, in which the molecular defect was shown to be a mutation in the ATP binding cassette transporter 1 (ABCA1) gene. It is currently thought that ABCA1 at the plasma membrane functions in cellular lipid efflux in the presence of apo A-I [22–26]. The fibroblasts from Tangier disease patients have a marked defect in the efflux of cholesterol to apo A-I [27]. In human embryonic kidney transfected HEK-293 cells, ABCA1 expression markedly increased cellular efflux to apo A-I. The caveolin-1 and ABCA-1 are expressed coordinately in differentiated THP-1 cells [17]. Both of them are involved in the enrichment with cholesterol of HDL and promote cellular cholesterol efflux. However, the cellular distribution of ABCA1 and caveolin-1, and the interaction between these two proteins in the HDL-mediated lipid efflux are still unknown.

Aortic endothelial cells represent one of the major cell types involved in atherogenesis. The aortic endothelium interacts continuously with plasma lipoproteins and endothelial–lipoprotein interactions have direct relevance to atherogenesis [28]. The accumulation of excess cholesterol in these cells could be reversed by HDL-mediated removal of excess cholesterol and transport of the cholesterol back to the liver. This is crucial in preventing the formation of atherosclerotic plaques in the arterial wall. In this study, we used confocal microscopy and immunoprecipitation analysis to investigate the location and the interaction between ABCA-1 and caveolin-1 in cholesterol-loaded endothelial cells after HDL exposure. Our results indicated that ABCA1 was colocalized with caveolin-1 and HDL on the cell surface. The ABCA1 directly interacted with HDL and caveolin-1. However, no direct interaction was found between HDL and caveolin-1.

## 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) supplemented with 10% fetal bovine serum and 50 U/ml penicillin–streptomycin under 5% CO<sub>2</sub>/95% air atmosphere. In order to establish pure endothelial cell cultures, ring explants were removed after 3–4 days of culture [29]. 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.

**Trypsin treatment and Western blotting.** The cholesterol-loaded cells were washed three times with phosphate-buffered-saline (PBS)–albumin, and then incubated at 4 °C for 2 h or at 37 °C for an additional 15 min with FAFA–DMEM containing 50 µg/ml HDL.

For the control, non-cholesterol-loaded cells were first incubated with lipoprotein deficient serum for 24 h before incubation with FAFA-medium containing 200 µg/ml LDL as described above. In order to determine if the lipoprotein particles were internalized by cells, the cells were washed twice with PBS–albumin at 4 °C and incubated with ice-cold trypsin for 8 min to release cell surface bound proteins [30]. For analysis of cell-associated proteins after trypsin treatment, the cells were washed with medium containing 2 mg/ml soybean trypsin inhibitor to quench trypsin activity and solubilized in Tris–HCl, 10 mM, pH 7.4, containing 150 mM NaCl, 1 mM benzamidine, 0.5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride dissolved in dimethyl sulfoxide. Total cellular proteins were separated by 12% SDS–PAGE for HDL and 8% SDS–PAGE for LDL, respectively, and electrotransferred to PVDF membrane. Blots were probed with goat anti-human HDL (1:1500 dilution) (Intra Cell, USA), rabbit anti-human LDL (1:7500 dilution) (Intra Cell, USA) or mouse anti-human β-actin (1:20,000 dilution) (Sigma, USA) primary antibody, and the appropriate HRP-conjugated secondary IgG antibodies were visualized using enhanced chemiluminescence reagent (NEN, USA).

**Localization of HDL and endosome markers by immunofluorescence microscopy.** Cholesterol-loaded aortic endothelial cells grown on a coverslip 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–DiI and 2 mg/ml FAFA at 37 °C for 15 min. The cells were then chilled on ice, washed three times with ice-cold PBS–albumin, fixed in cold methanol for 5 min and air-dried. After washing with PBS, coverslip cultures were incubated with early endosome marker CD71 (1:100, v/v), late endosome marker LAMP2 (1:200, v/v) and rabbit polyclonal antibodies at 37 °C for 30 min, then washed three times with PBS and incubated for 30 min with FITC-conjugated goat anti-rabbit IgG (1:300, v/v). Coverslips were washed three times in PBS, mounted on slides, and photographed with a LSM 510 confocal microscope (Zeiss, Germany).

**The expression of ABCA1 and caveolin-1 by HDL.** The cholesterol-loaded cells grown in 75 cm<sup>2</sup> flasks for 48 h were washed three times with PBS–albumin and incubated with DMEM containing 50 µg/ml HDL and 2 mg/ml FAFA at 37 °C for various times (5, 15 min, 1, 3, 6, 12, 24, and 48 h). Cells were harvested at each time interval and the total proteins were separated by 8% SDS–PAGE and electrotransferred to PVDF membrane. Blots were probed with rabbit anti-human ABCA1 (1:1500 v/v) (Novus Biologicals, USA), rabbit anti-human caveolin-1 (1:1500 dilution) (Intra Cell, USA), and mouse anti-β-actin (1:10,000 v/v, Sigma, USA) primary antibody, and the appropriate HRP-conjugated secondary IgG antibodies were visualized using enhanced chemiluminescence reagent (NEN, USA). The intensity of reaction bands was analyzed by an Image Gauge system (Fuji, Japan).

**Localization of ABCA1, caveolin-1, and HDL by confocal microscopy.** Cholesterol-loaded cells grown on a coverslip 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–DiI and 2 mg/ml FAFA at 37 °C for 15 min. The cells were then chilled on ice, washed three times with ice-cold PBS–albumin, fixed in cold methanol for 5 min, and air-dried. After washing with PBS, coverslip cultures were incubated with mouse anti-human caveolin-1 antibody (1:200, v/v) (Santa Cruz, CA) and rabbit anti-human ABCA1 antibody (1:50, v/v) for 30 min. Coverslips were then washed three times with PBS, and incubated for 30 min with FITC-conjugated goat anti-rabbit IgG (1:200, v/v) followed by Cy<sub>5</sub> conjugated anti-mouse IgG (1:200, v/v). Coverslips were washed three times in PBS, mounted on slides, and photographed with a LSM 510 confocal microscope (Zeiss, Germany). A 3D view was composed from Z-section scanning images and analyzed by the LSM 510 image system (Zeiss, Germany).

**Chemical cross-linking and immunoprecipitation analysis.** Cells grown on a 25 cm<sup>2</sup> flask were incubated with HDL (50 µg/ml)

medium at 37 °C for 1 h. Cells were then placed on ice for 15 min and washed three times with PBS. Dithiois (DSP) (Sigma, USA) was dissolved in dimethyl sulfoxide immediately before use and diluted to 250  $\mu$ M with PBS, and 5 ml was added in the flask. Cells were incubated at room temperature for 1 h, the medium was removed, and the cells were washed twice with PBS [24]. Cells were scraped and lysed at 4 °C with lysis buffer (Tris-HCl, 10 mM, pH 7.4, containing 150 mM NaCl, 1 mM benzamide, 0.5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride dissolved in dimethyl sulfoxide). The immunoprecipitation was performed with a reversible immunoprecipitation system kit (Upstate, USA). In brief, a 500- $\mu$ l aliquot of diluted cell lysate (500  $\mu$ g) was incubated with 2  $\mu$ l (4  $\mu$ g) goat-anti-apo A-I antibody or 10  $\mu$ l (4  $\mu$ g) rabbit anti-caveolin-1 antibody and 10  $\mu$ l (1  $\mu$ g) antibody capture affinity ligand on a rocking platform at room temperature for 15 min. The lysate and antibody mixture were transferred to an affinity spin column and centrifuged for 5–10 min at 1500g (4200 rpm). After centrifugation, the final “flow through” samples were tested in Western blot analysis as the controls. The spin column was washed with release lysis/wash buffer and IP elution buffer. The eluted proteins and the control samples were loaded onto 12% SDS-polyacrylamide gel for electrophoresis followed by electrotransfer to a PVDF membrane. Western blots were probed with goat anti-human HDL (1:7500, v/v) (Intra Cell, USA), rabbit anti-human caveolin-1 (1:7500, v/v), and rabbit anti-human ABCA1 (1:1000, v/v) primary antibody. The appropriate HRP-conjugated secondary IgG antibodies were visualized using enhanced chemiluminescence reagent (NEN, USA).

## Results

### Trypsin treatment and Western blot analysis

When cholesterol-loaded cells were incubated with HDL for 2 h at 4 °C, the HDL apoprotein was removed by trypsin treatment (Fig. 1A). When cells were re-

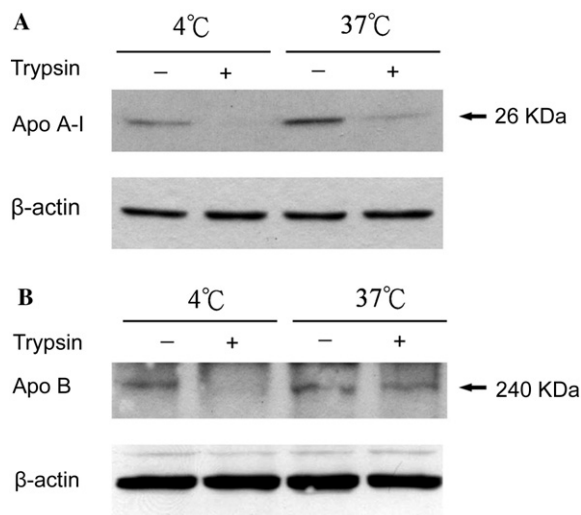


Fig. 1. Western blot analysis showed that when cholesterol-loaded endothelial cells were incubated with HDL at 4 °C for 2 h, or 37 °C for an additional 15 min, HDL was detected (A). When cells were incubated at 4 or 37 °C followed by trypsin digestion, only a trace of HDL remained associated with cells, while LDL was readily detected in the cell lysates after trypsin treatment (B).

warmed to 37 °C for an additional 15 min, there was a marked increase in the amount of apoprotein that became associated with cells. However, when cells were incubated at 4 or 37 °C followed by trypsin digestion, all of these apoproteins were removed from cells by trypsin-treatment (Fig. 1A). These results provide further evidence that the HDL particles are not internalized to any significant extent during 15 min incubation at 37 °C. This lack of internalization of apo A-I was evident under conditions where internalization of LDL was readily detected (Fig. 1B).

### The location of HDL and endosomal markers in cholesterol-loaded endothelial cells

In our previous study, HDL colocalized with caveolin-1 on the cell surface or in the plasmalemmal vesicles and invaginations, and no free HDL was revealed in the cytoplasm [22]. In this study, HDL did not colocalize with either CD71, an early endosome marker, or LAMP2, a late endosome marker (Fig. 2). These results further demonstrated that HDL-mediated cholesterol efflux was not associated with either early or late endocytic compartments.

### The expression of ABCA1 and caveolin-1 in cholesterol-loaded endothelial cells after HDL incubation

The Western blot analysis showed that when cells were incubated with cholesterol for 48 h, the expression of ABCA1 significantly increased compared with non-cholesterol-loaded cells (Fig. 3). When the cholesterol-loaded cells were incubated with HDL, the level of ABCA1 remained high during the first 3 h and gradually decreased after 6 h of HDL incubation (Fig. 3). The expression of caveolin-1 increased during the first 1 h and decreased after 3 h of HDL incubation.

### Colocalization of ABCA1 and caveolin-1 in cholesterol-loaded endothelial cells after HDL incubation

The localization of ABCA1 and caveolin-1 in cholesterol-loaded endothelial cells after HDL incubation was performed by immunofluorescence confocal microscopy. The results showed that when the endothelial cells were incubated in the culture medium with cholesterol for 48 h, the HDL, ABCA1, and caveolin-1 were revealed in a similar distribution pattern (Figs. 4A–C). HDL colocalized with ABCA1 (Fig. 4D) and caveolin-1 (Fig. 4E). ABCA1 also colocalized with caveolin-1 (Fig. 4F). The merged image showed the colocalization of HDL, ABCA1, and caveolin-1 (Fig. 4G). The 3D confocal images of the top view showed that the HDL colocalized with ABCA1 and caveolin-1 predominantly on the cell surface (Figs. 4H and I).

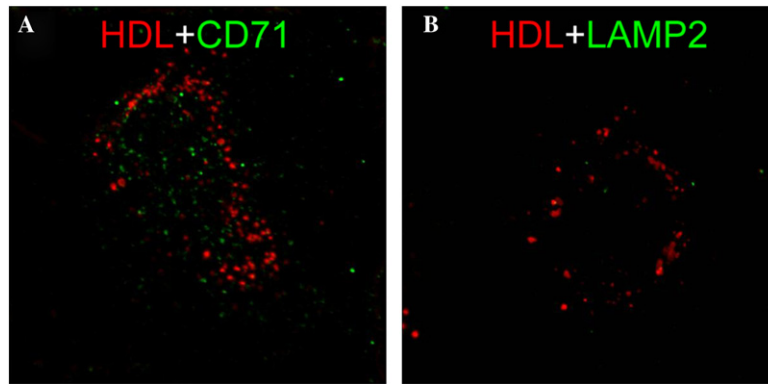


Fig. 2. A cholesterol-loaded endothelial cell showing the HDL (red fluorescence) did not colocalize with either (A) early endosome marker CD71 (green fluorescence) or (B) the late endosome marker LAMP2 (green fluorescence). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper).

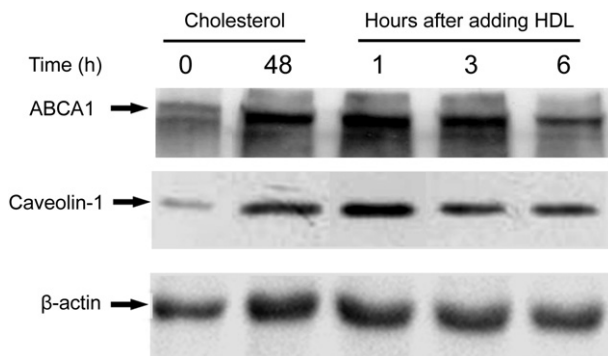


Fig. 3. The Western blot analysis showed that when cells were incubated with cholesterol for 48 h, the expression of ABCA1 and caveolin-1 significantly increased compared with that of non-cholesterol-loaded cells. These levels gradually decreased after 3 h of HDL incubation.

*ABCA1 directly interacts with HDL and caveolin-1 but caveolin-1 does not interact directly with HDL*

In order to clarify whether there is a direct interaction between HDL, caveolin-1, and ABCA1, chemical cross-linking and immunoprecipitation were performed. The results indicated that ABCA1 was co-immunoprecipitated with HDL and caveolin-1 from the cell lysates (Fig. 5). In contrast, caveolin-1 was not co-immunoprecipitated with HDL. These results demonstrate that ABCA1 directly interacts with HDL, and ABCA1 directly interacts with caveolin-1, but caveolin-1 does not directly interact with HDL.

## Discussion

Multiple studies conducted over the past decade have indicated that caveolae are the major membrane domains facilitating the transport of excess cholesterol to HDL on the cell surface of aortic endothelial cells [13]. Caveolin-1, the main structural protein component of caveolae, regu-

lates this process because down-regulation of caveolin-1 decreases cellular cholesterol efflux [11,17]. In supporting these data, in vivo expression of caveolin-1 in the mouse liver causes an increase in plasma HDL cholesterol level [15]. It appears that caveolin-1 enhances the availability and delivery of cholesterol to caveolae for efflux. At present, however, the cellular mechanism of the efflux process from caveolae to HDL is not known. A mutation ABCA1 was recently identified as the cause of Tangier disease, a condition associated with very low HDL, cholesteryl ester accumulation in tissue macrophages, and an apparent increased risk of atherosclerotic cardiovascular disease. ABCA1 facilitates the addition of phospholipids and cholesterol to free apo A-I, initiating the formation of HDL [31]. However, the interaction between caveolin-1, ABCA1, and HDL on the cell surface is still a mystery. This paper reports findings concerning the morphological and biochemical aspects of the relationship between ABCA1, caveolin-1, and HDL. Our results provide evidence for a direct interaction between ABCA1 and HDL, ABCA1, and caveolin-1, but not HDL and caveolin-1 in cholesterol-loaded endothelial cells.

It was postulated that HDL enhancement of cholesterol efflux is accomplished by the tethering of the HDL particle to the membrane. Some progress has been made recently in the identification of HDL binding proteins, but the exact receptor structure is still unclear. 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 [32]. Besides SR-BI, ABCA1 has been advanced as a putative HDL receptor [31]. Fielding et al. [33] suggested that the complex of apo A-I and phospholipids is a much better acceptor of free cholesterol than apo A-I itself. In this study, the HDL, ABCA1, and caveolin-1 show colocalization by confocal microscopy. The chemical cross-linking and immunoprecipitation analysis indicated that there is a protein/protein interaction between ABCA1 and apo A-I, the major protein in

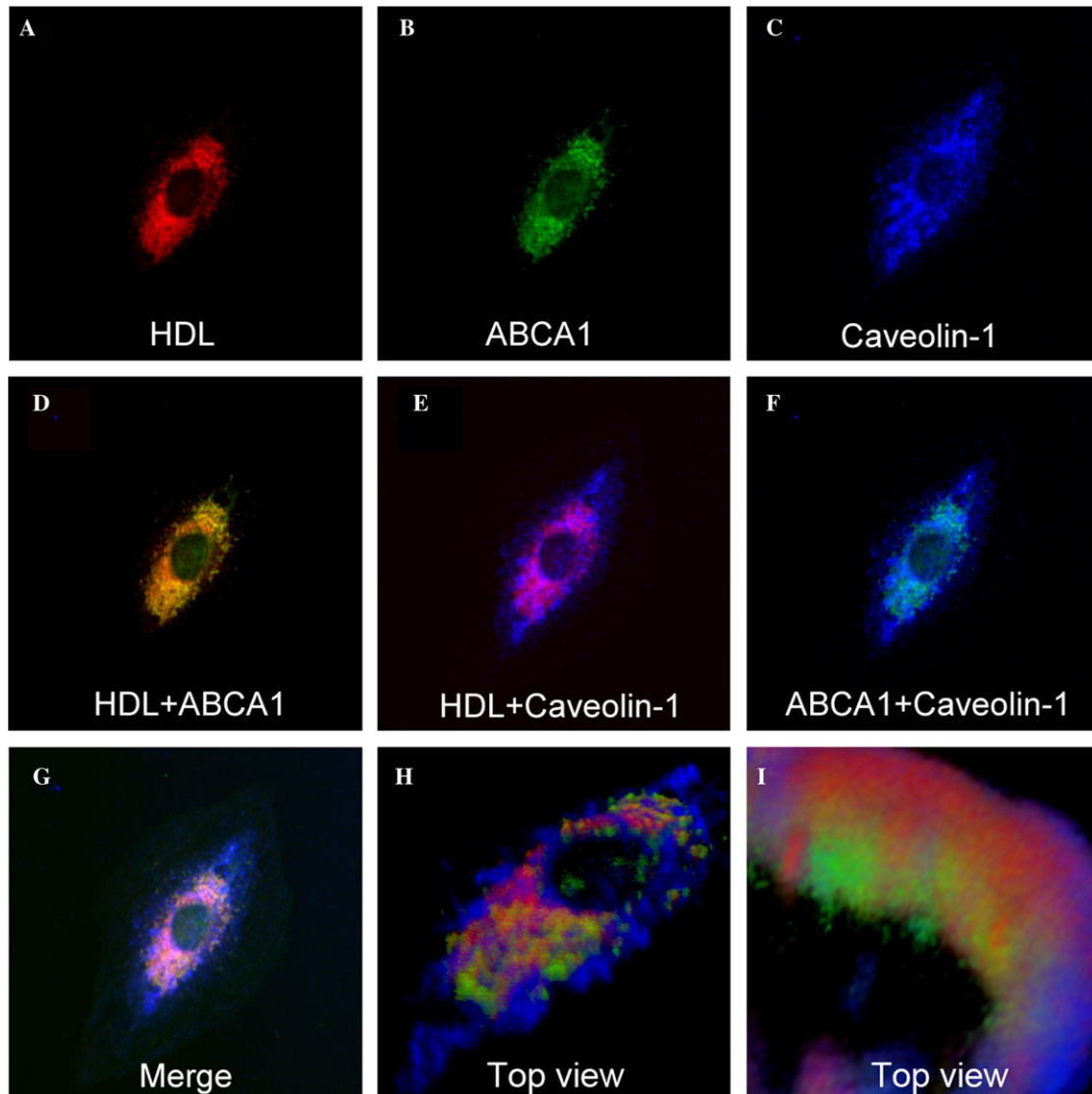


Fig. 4. The confocal images of an endothelial cell stained with HDL, ABCA1, and caveolin-1. The red fluorescence, green fluorescence, and blue fluorescence indicate the location of HDL, ABCA1, and caveolin-1, respectively (A–C). HDL and ABCA1, HDL and caveolin-1, ABCA1 and caveolin-1 colocalize, respectively (D–F). HDL, and ABCA1, and caveolin-1 also show colocalization (G). A top view of the 3D confocal image (H) and an enlarged image (I) show that the HDL (red) colocalized with ABCA1 (green) and caveolin-1 (blue) on the cell surface.

HDL, as well as ABCA1 and caveolin-1. However, caveolin-1 does not interact directly with HDL. We propose that ABCA1 resides on the caveolae membrane and interacts with HDL for cholesterol efflux (Fig. 6). Further research will be required to establish additional details of the interrelationships among these proteins.

Two different pathways have been suggested for HDL-mediated cholesterol efflux: (i) a docking receptor promoting cholesterol translocation of cholesterol from the intracellular cholesterol pool to the cell membrane through activation of phospholipases and protein kinase C [34,35], or (ii) a receptor-mediated intracellular endosomal pathway termed “retroendocytosis” [9,36]. We have previously demonstrated that most of the HDL colocalized with caveolin-1 on the cell surface or in

invaginations into the cytoplasm [20]. In this study, the HDL did not colocalize either with early endosome or late endosome marker in the cholesterol-loaded endothelial cells. We analyzed the distribution pattern of HDL and caveolin-1 in the presence of the endocytotic inhibitor, monensin, but observed no appreciable difference between treated and untreated cells (unpublished data). Furthermore, the HDL apoproteins were removed from cells by trypsin-treatment. This lack of endocytosis of HDL in the cells was evident under assay conditions where internalization of LDL was readily detected. These results provide further evidence that the HDL particles are not internalized to any significant extent during HDL incubation. A similar result has also been reported with HDL binding to its receptor on

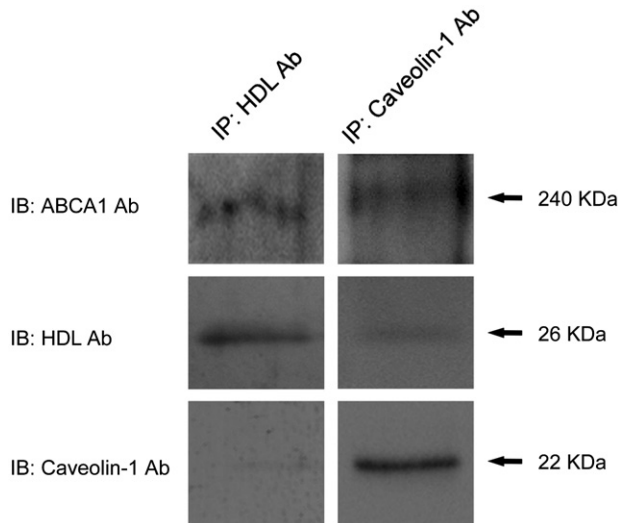


Fig. 5. The cholesterol-loaded endothelial cells were incubated with HDL, followed by chemical cross-linking and immunoprecipitation with HDL, ABCA1, and caveolin-1 as described in Materials and methods. The ABCA1, HDL, and caveolin-1 were detected by Western blot. When the lysates were immunoprecipitated with HDL antibody, the HDL and ABCA1 were abundant in the precipitated proteins. When the lysates were immunoprecipitated with caveolin-1 antibody, the caveolin-1 and ABCA1 were abundant in the precipitated proteins.

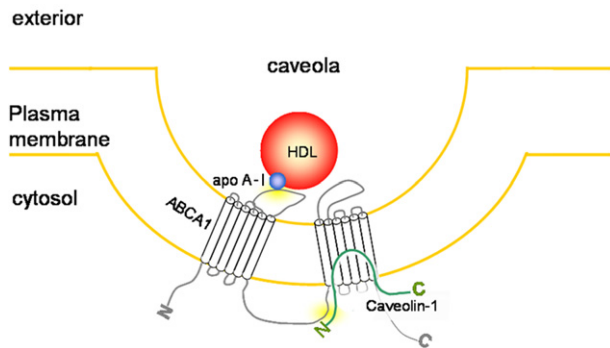


Fig. 6. Model illustrating the structural relationships of ABCA1 and caveolin-1 on a plasma membrane caveola during HDL-mediated cholesterol efflux.

human fibroblasts and mouse peritoneal macrophages without being internalized into cellular compartments [30,35]. The small amount of cell-associated HDL that was resistant to trypsin treatment appeared to represent a receptor-independent interaction.

It is currently thought that ABCA1 at the plasma membrane functions in cellular lipid efflux in the presence of HDL and apo A-I. [23–25]. The increased lipid efflux appears to involve a direct interaction between apo A-I and ABCA1 [23]. Enhancement of cholesterol efflux by over-expression of ABCA1 in the liver also led to a significant increase in plasma concentrations of HDL. Higher plasma HDL levels may result in

enhanced protection against atherosclerosis either due to enhanced RCT or due to other antiatherogenic properties of HDL. In this study, we demonstrated that HDL colocalized with both caveolin-1 and ABCA1 on the cell surface. The immunoprecipitation analysis indicated that ABCA1 directly interacts with both caveolin-1 and apo A-I, the major protein component in the HDL. ABCA1 may be involved in the plasmalemmal caveolae structure following the docking pathway to remove the lipids from cells, a process that may well be relevant for treating atherosclerosis.

The effects of ABCA1 and caveolin-1 might be additive, providing even higher protection against atherosclerosis. Over-expression of caveolin-1 enhanced the expression of ABCA1 and increased cholesterol efflux. The colocalization of HDL and ABCA1 was disturbed in caveolin-1 down-regulated cells (unpublished data). Apparently, caveolin-1 is necessary for interaction between ABCA1 and HDL. Whether aortic endothelial cells export cholesterol via the ABCA1 pathway using apo A-I/HDL as acceptors needs further investigation. In this study, the levels of expression of ABCA1 and caveolin-1 increased significantly after 48 h of cholesterol incubation. Unlike the situation in rat aortic endothelial cells, expression was not up-regulated in either HUVEC or HAEC [33] when the cells were free cholesterol-loaded. More experiments need to be done to explain these disparate observations.

## Acknowledgment

This research was supported by Grant NSC-92-2311-B-029-005 from the National Science Council, Taiwan, Republic of China.

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