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# THE FUNCTION OF ALBUMIN AND LIPOPROTEINS CONTAINING APOA-I IN PROMOTING CELLULAR CHOLESTEROL EFFLUX FROM HUMAN SKIN FIBROBLASTS

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# ABSTRACT

This study is designed to elucidate the factors that control the transport of cholesterol molecules from the plasma membrane of cultured human cells to lipoprotein particles. We are particularly interested in determining how the composition of HDL particles and their related physico-chemical parameters control the ability of these lipoproteins to release cellular cholesterol. We have investigated the ability of both serum albumin and well defined, homogeneous reconstituted HDL particles (LpA-I) to receive and retain cholesterol molecules from cultured human fibroblasts.

Human serum albumin induces a time-dependent bi-phasic efflux of cholesterol from non-cholesterol loaded human skin fibroblasts. Although the magnitude of transfer to albumin is lower than that to reconstituted discoidal LpA-I, it is specific and occurs within a normal physiological concentration range. The reverse transfer of albumin-bound cholesterol back to fibroblasts is very rapid and is inhibited by both LDL and LpA-I, which appear to be able to compete for the transferred lipids. Therefore, albumin appears to be able to contribute to the net efflux of cellular cholesterol and its transfer to lipoprotein particles.

Model discoidal and spherical LpA-I particles have been prepared in vitro by the complexation of apoA-I and pure lipids using cholate dialysis or co-sonication method respectively. LpA-I surface lipid composition; phosphatidylcholine (PC), cholesterol (UC), sphingomyelin (SM) and phosphatidylinositol (PI), and core neutral lipid composition; cholesteryl esters and triglycerides, have been varied systematically to characterize the effect of each constituent on the release of cellular cholesterol and on LpA-I physico-chemical properties. The efflux to discoidal LpA-I is independent of PC or UC content, but

is related to the molecule number of apoA-I per LpA-I particle and to the accompanying changes in particle sizes. The addition of PI and SM into Lp2A-I significantly increases the efflux of cholesterol to these particles, apparently by modifying the interfacial lipid packing and surface charge. In sonicated LpA-I complexes, the association of few molecules of PC and SM transforms lipid-free apoA-I into small distinct lipoprotein-like complexes with only one apoA-I (Lp1A-I), which are significantly better acceptors of cellular cholesterol than lipid-free apoA-I. The progressive increase of PC from 5 to 35.5 moles per apoA-I alters the α-helix content of apoA-I and the negative surface charge of the LpA-I, and significantly increases the ability to receive and retain cholesterol molecules from the cultured cells. In contrast, at high PC/apoA-I ratio, inclusion of either SM or PI or core neutral lipids into a sonicated Lp2A-I has no effect on cholesterol efflux.

In conclusion, this study shows that efflux of cellular cholesterol is mediated differently by albumin, discoidal or spherical LpA-I. While particle size and surface lipid physical properties appear to be critical to the efflux of cholesterol to discoidal LpA-I, the ability of sonicated LpA-I to receive and retain cellular cholesterol is dependent on factors that modify the conformation and stability of apoA-I. The study shows that the progressive association of PC and SM with apoA-I is critical to transform this apolipoprotein into an optimal recipient of cholesterol molecules.

# DEDICATION

This thesis is dedicated to Benzi, Gary and Connie.

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| | |
|---|---|
| ACAT : | acylCoA: cholesterol acyltransferase |
| apoA-I | apolipoprotein A-I |
| apoE | apolipoprotein E |
| apoA-II | apolipoprotein A-II |
| apoIV | apolipoprotein A-IV |
| apoC | apolipoprotein C |
| apoB | apolipoprotein B |
| apoCs | apolipoprotein containing all the isoforms of apoC |
| apoD | apolipoprotein D |
| BSA | bovine serum albumin |
| cAMP | cyclic adenyl monophosphate |
| CAD | coronary artery disease |
| CD | circular dichroism |
| CE | cholesteryl ester |
| CETP | cholesterol ester transfer protein |
| DAG | diacylglycerol |
| DC | duty cycle |
| DMEM | Dulbecco's modified Eagle's medium |
| DMPC | dimyristoryl phosphatidylcholine |
| DMS | dimethylsuberimidate |
| EYPC | egg yolk phosphatidylcholine |
| FBS | fetal bovine serum |
| HSA | essentially fatty acid-free human serum albumin |
| HDL | high density lipoprotein |
| HMG-CoA reductase | 3-hydroxy-3-methylglutaryl coenzyme A |
| H-TGL | hepatic triacylglyceride lipase |
| IDL | intermediate density lipoproteins |
| $IP_3$ | inositol triphosphate |
| KBr | potassium bromide |
| LCAT | lecithin:cholesterol acyltransferase |
| LDL | low density lipoprotein |
| LpA-I | lipoprotein containing only apoA-I |
| LpA-I/A-II | lipoprotein containing both apoA-I and apoA-II |
| Lp1A-I | LpA-I containing 1 mole of apoA-I per particle |
| Lp2A-I | LpA-I containing 2 moles of apoA-I per particle |
| Lp3A-I | LpA-I containing 3 moles of apoA-I per particle |

| | |
|---|---|
| Lp4A-I | LpA-I containing 4 moles of apoA-I per particle |
| LpA-IV | lipoproteins containing only apoA-IV and migrating to a slow α-position" on agarose gel electrophoresis |
| LPL | lipoprotein lipase |
| γ-LpE | lipoproteins containing only apoE and migrating to a γ-position on agarose gel electrophoresis |
| nCEH | neutral CE hydrolase |
| MLV | multilamellar vesicles |
| NAR | Nagase analbuminemic rats |
| NGGE | non-denaturing gradient gel electrophoresis |
| nHDL | nascent HDL |
| PBS | phosphate buffered saline |
| PC | phosphatidylcholine |
| PE | phosphatidylethanolamine |
| PL | phospholipid |
| PLTP | phospholipid transfer protein |
| POPC | 1-palmitoyl 2-oleoyl phosphatidylcholine |
| PI | phosphatidylinositol |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PMA | phorbol myristic acetate |
| Pre-β-HDL | a species of HDL that migrates to a pre β position on agarose gel electrophoresis |
| PS | phosphatidylserine |
| PVP | polyvinylpyrrolidone |
| RCT | reverse cholesterol transport |
| SDS/PAGE | sodium dodecylsulfate/polyacrylamide gel eletrophoresis |
| SM | sphingomyeline |
| SMase | sphingomyelinase |
| TBS | Tris-base buffered saline |
| TC | total cholesterol |
| TG | triglycerides |
| TNM | tetranitromethane |
| UC | unesterified cholesterol |
| VLDL | very low density lipoprotein |

# GENERAL INTRODUCTION

## 1. High Density Lipoproteins and Reverse Cholesterol Transport

Atherosclerosis and coronary artery disease (CAD), characterized by the deposition of lipids and cholesterol in aortic or coronary arteries, have been the leading cause for the mortality in North America. Although the etiology of this disease is very complicated and involves multiple factors, high density lipoprotein (HDL) has been well accepted as an anti-atherogenic lipoprotein for its function in "reverse cholesterol transport" (RCT), a process in which cholesterol is desorbed from extrahepatic cells and transported to the liver for catabolism. This pathway as indicated in Fig. 1 plays a central role in the transfer/metabolism of cholesterol. Many studies have demonstrated a significant negative correlation between plasma HDL/HDL-cholesterol levels and the incidence of CAD (Kunkita et al., 1985, Puchois et al., 1987, Cheung et al., 1991, Funz et al., 1994). Patients with defect in HDL metabolic pathways, such as lecithin:cholesterol acyltransferase (LCAT) deficiency (Glomset et al., 1973), apoA-I gene mutations (Lackner et al., 1993, Karathanasis et al., 1983), familial apoA-I and apoC-III deficiencies (Norum et al., 1982, Schaefer et al., 1985) and Tangier disease (also known as familial HDL-deficiency) (Walter et al., 1994, Serfaty-Lacroshiere et al., 1994, Francis et al.,

1995), showed defective removal of cholesterol from cells although they were prone to premature atherosclerosis to different extent.

The concept of RCT, originally introduced by Glomset, J. A. (1968), indicates a process that consists of a series of metabolic events. As shown in Fig. 2, this process involves the efflux of free cholesterol from extrahepatic tissues to HDL, the esterification of the cell-derived cholesterol in HDL by LCAT, the subsequent transfer of the esterified cholesterol from HDL to triglyceride-rich lipoproteins by the action of cholesterol ester transfer protein (CETP), and the delivery of cholesterol to the liver by the uptake of either HDL itself or via other lipoproteins such as very low density lipoprotein (VLDL) or low density lipoprotein (LDL) by apoE or apoB/E receptors. As the first step in the process, the efflux of free cholesterol from extrahepatic cells to HDL is usually the rate limiting step. The postulate of the RCT pathway opened a new field for the study of HDL and led to extensive investigations in the past three decades, which have been reviewed by several main investigators from different perspectives (Fielding et al., 1992, 1995a, Reichl, 1994, Rothblat et al., 1991, 1992, Johnson et al., 1991, Phillips et al., 1987).

It is clear that cellular cholesterol efflux driven by HDL contributes directly to the cholesterol homeostasis of extrahepatic cells. As showed in Fig. 3, cholesterol is able to enter extrahepatic cells by three pathways: the uptake of

LDL by LDL receptors, direct phagocytosis of extracellular lipid droplets or lipoproteins, and the uptake of modified LDL via scavenger LDL receptors in some cells such as macrophages. Cholesterol ester (CE) generated from the esterification of unesterified cholesterol (UC) by acyl CoA:cholesterol transferase (ACAT) is the storage form of cholesterol in extrahepatic cells. It has been well known that extrahepatic cells are unable to catabolize cholesterol *in situ* (Bernard et al., 1990, Scobey et al., 1989). In most cases, sterol in these cells has to be hydrolyzed first into UC, then to be transferred to the cell surface, and to be released from there to appropriate extracellular acceptors. Therefore, efflux is the exclusive pathway to balance the exogenous uptake and endogenous synthesis of cholesterol, thus HDL plays an important role in clearing the cholesterol accumulated in extrahepatic cells, and in maintaining cellular cholesterol homeostasis.

## 2. *In Vivo* Evidence for the Function of HDL in Reverse Cholesterol Transport

The function of HDL in RCT/anti-atherogenic process has been well demonstrated by many *in vivo* studies. HDL administrated to rabbits fed with high-cholesterol chow was able not only to inhibit the formation and cholesterol-deposition of aortic fatty streaks (Badimon et al., 1989), but also to induce the

regression of the established aortic fatty streaks (Badimon et al., 1990). After intravenous injection of acetylated or native LDL to particularly label and load cholesterol in peripheral tissues, cholesterol clearance studies have indicated that HDL, especially the d=1.175-1.21 g/ml fraction, was the principle acceptor for the tissue-derived cholesterol (Miller et al., 1985). Similar results were also observed when whole blood or serum was used to perfuse spleen from rats which had been injected with $^{3}$H-cholesterol-loaded erythrocytes (Mindham et al., 1990, 1991).

Production of transgenic mice has made it possible to observe directly the relationship between HDL apolipoprotein components and atherogenesis. Transgenic mice with overexpression of human apoA-I and HDL (Rubin et al., 1991a) were resistant to diet-induced atherosclerosis and were protected from the development of fatty streak lesions (Rubin et al., 1991b, Schultz et al., 1993). In contrast, apoE knockout mice showed severe hypercholesterolemia and developed atherosclerosis rapidly (Plump et al., 1992 , Zhang et al., 1992). The introduction of a human apoA-I gene into the hypercholesterolemic apoE knockout mice reduced significantly the susceptibility of the mice to atherosclerosis, as compared to the mice with apoE knockout but without apoA-I overexpression (Pászty et al., 1994). In contrast, transgenic mice with overexpression of apoA-II showed increased HDL-cholesterol level, but promoted rather than retarded aortic fatty streak development (Warden et al., 1993, Mehrabian et al., 1993).

In spite of the difficulty of *in vivo* studies in human subjects, there is some experimental evidence that supports the presence of HDL-mediated RCT. The prior accessibility of HDL to tissue-derived cholesterol was showed in subjects whose tissue cholesterol was pre-labelled by intravenous injection of $^{14}C$-cholesterol, where the radioactive cholesterol was transferred preferentially from tissues to the HDL of lymph draining those tissues, and then to both HDL and LDL (Reichl et al., 1980). In addition, HDL was also the predominant carrier for tissue-derived cholesterol in the plasma of subjects who were undergoing extensive body weight loss by caloric restriction (Nestel and Miller, 1978). In cultured cells including human skin fibroblasts (Ranganathan et al., 1989), human EA.hy926 endothelial cells (Kilsdonk et al., 1993b) or cholesterol-ester loaded J774 macrophages attached to microcarrier beads (Michishita and Thompson, 1991, Nakamura et al., 1993), VLDL, LDL or HDL were all able to release radioactive cellular cholesterol; however, only HDL resulted in a net removal of cellular cholesterol. Therefore, the function of HDL in releasing cellular cholesterol is unique to other species of lipoproteins.

## 3. The Heterogeneity of HDL and Reconstituted HDL-like Particles

HDL is referred to as the lipoprotein fraction within the density range of 1.063 - 1.210 g/ml by potassium bromide (KBr) density gradient

ultracentrifugation, a traditional method for the separation of lipoproteins. This fraction represents a group of lipoprotein particles heterogeneous in size, density, composition, as well as other physico-chemical characteristics.

### *3.1. The Heterogeneity of Plasma HDL*

#### 3.1.1. Size and apolipoprotein composition of plasma HDL

Plasma HDL within the density range mentioned above could be divided into $HDL_2$ (d=1.063-1.125 g/ml) and $HDL_3$ (1.125-1.21 g/ml) respectively. Two subspecies of $HDL_2$ (10.6 & 9.2 nm) and three subspecies of $HDL_3$ (8.4, 8.0 & 7.6 nm) have been identified by non-denaturing gel electrophoresis (NDGGE) (Blanche et al., 1981, Williams et al., 1990). Five similar subspecies of HDL particles with hydrated diameters of 12.2, 11.0, 9.8, 8.7 and 7.6 nm were also identified when either whole serum or Schmitz ultracentrifugally isolated HDL was analyzed by a combined method of HPLC using gel permeation columns and selective detection of cholesterol or choline-containing phospholipids (Okazaki et al., 1982). Recently, HDL has been reported to contain 12 subspecies of particles by two-dimension gel electrophoresis (Asztalos et al., 1993a).

There are at least eight apolipoproteins that are associated with HDL in various proportions, such as apoA-I, apoA-II, apoE, apoC-I, apoC-II, apoC-III,

apoA-IV and apoD (Cheung et al., 1984, James et al., 1988, Vézina et al., 1988, Bekaert et al., 1991, Kunitake et al., 1994). ApoA-I and apoA-II are the two predominant apolipoproteins in HDL, which form LpA-I and LpA-I/A-II, the two main subspecies of HDL in plasma (Cheung et al., 1984, James et al., 1989). Earlier reports have indicated two distinct populations of LpA-I with Stoke's diameters of 10.8 and 8.5 nm and containing 4 and 3 apoA-I per particle respectively (Nichols et al., 1985). Recently, however, a report has indicated the presence of even smaller LpA-I particles (7.5-7.7 nm) in normolipidemic plasma (Duverger et al., 1993, Ohta et al., 1995b). LpA-I/A-II subfraction usually consists of three species of particles at 8.0, 8.9 and 9.6 nm respectively (Cheung et al., 1984, 1988a). LpA-I binds more of plasma LCAT and CETP relative to LpA-I/A-II particles (Cheung et al., 1986, Kunitake et al., 1994).

3.1.2. The pre β–HDL in plasma

In addition to the major HDL particles that migrate to α- position on agarose gel electrophoresis, a minor subspecies of HDL exhibiting pre β electrophoretic mobility was identified several years ago from LpA-I isolated by immunoabsorption (Kunitake et al., 1985). It represents an HDL subspecies that is removable during ultracentrifugal isolation of HDL (Asztelos et al., 1993a). Pre β-HDL contains predominantly apoA-I which represents approximately 6-10% of the

total apoA-I of nomolipidemic plasma (Ishida et al., 1987, Castro & Fielding, 1988, Neary et al., 1991, Miida et al., 1992). When separated from fresh plasma, pre β–HDL could be resolved into three major subpopulations as pre $\beta_1$, pre $\beta_2$ and pre $\beta_3$ on two dimensional gel electrophoresis (Castro & Fielding, 1988, Francone et al., 1989, 1990b, Miida et al., 1990).

The smallest and most abundant pre $\beta_1$-HDL is a lipid-poor particle, which has a molecular weight of 60-70 kDa, contains one mole of apoA-I per particle as sole protein, and has a calculated diameter of 5-6 nm. This subspecies of HDL particles contains 10-40% of phospholipids (PL) with lecithin and sphingomyelin (SM) in almost equimolar concentrations (Fielding and Fielding, 1995a). The tertiary structures of these small HDL particles are distinct from discoidal pre $\beta_2$-HDL and spherical α-HDL as indicated by their accessibility to certain specific monoclonal antibodies (Fielding et al., 1994). The pre $\beta_2$-HDL has an apparent molecular weight of 300 kDa with a composition similar to that of a discoidal particle containing 3 apoA-I per particle. It is rich in lecithin and contains a small proportion of SM and UC but no CE (Fielding and Fielding, 1995a). The largest pre $\beta_3$-HDL particles are associated apparently with LCAT, CETP and other apolipoproteins such as apoD, and have sizes larger than that of α-HDL (Fielding et al., 1992, Francone et al., 1989). The pre $\beta_1$-HDL usually contains a major and

a minor component, and the pre $\beta_2$-HDL consists of three to five subspecies Francone et al., 1990b, 1989, Miida et al., 1990, Castro & Fielding, 1988). Significantly elevated level of pre β HDL have been found in familial hypercholesterolemia, hypertriglyceridemia and congenital LCAT deficiency (Ohta et al., 1994a, b, 1995c, Ishida et al., 1987, Francone et al., 1989, Glomset et al., 1980).

3.1.3. The morphology of HDL

The majority of plasma HDL particles are in spherical shape with an apolar core of CE and triacylglycerol molecules encapsulated by a surface monolayer of phospholipids and apolipoproteins. Free cholesterol molecules are partitioned between the core and the surface, and most of them are on the surface (Lund-Katz et al., 1984) (Fig. 4). However, newly synthesized or nascent HDL (nHDL) appears to be large discoidal particles (Castel et al., 1991, McCall et al., 1988, 1989, Thrift et al., 1986), for which a similar model has been proposed by different laboratories since 1969 (see review of Brouillette and Anantharamaiah, 1995). The latest model suggested by Marcel and colleagues is shown in Fig. 5. According to this model, phospholipids form a disc bilayer with its hydrophobic fatty-acyl chains toward the lipid core and its hydrophilic moiety toward the aqueous phase. In larger LpA-I particles (9.6 nm), apoA-I is associated with the

edge of the PL disc with its 7-8 amphipathic α-helixes parallel to the acyl chain of the PL and to the disc axis, and adjacent amphipathic helixes are antiparallel to one another. The sequences between amino acid residues 121-165 and between 99-143 are relatively flexible, and stay as alternatively hinged domains in smaller particles (7.8 nm) (Calabresi et al., 1993). Cholesterol esterification induced by LCAT is known to result in the transfer of cholesterol from the surface to the core of HDL, thereby convert nascent discoidal HDL into mature spherical particles (reviewed by Jonas et al., 1991b).

### *3.2. Comparison of the Heterogeneity of Plasma HDL with Extravascular HDL*

Physiologically, tissue cells are bathed in interstitial fluid which is separated from plasma compartment by capillary endothelium. Therefore, lipoproteins in interstitial fluid are the first to interact with cell plasma membrane of extrahepatic cells and to accept cell-derived cholesterol. However, studies on cholesterol efflux to interstitial fluid lipoproteins are greatly limited by the difficulty in collecting interstitial fluid (review by Sloop et al., 1987). Consequently, lymph has frequently been used as a substitute and model for interstitial fluid in many experiments. Studies have demonstrated that HDL in lymph/interstitial fluid differs from plasma HDL in respect to a number of physico-chemical parameters, which are summarized as follows:

3.2.1. Composition of extravascular HDL

The composition of interstitial fluid/lymph depends greatly on the filtration capability of individual plasma components through the interstitium barrier. Generally speaking, interstitial fluid has a lower protein concentration with interstitial/plasma or lymph/plasma ratios of 0.09 for α-macroglobulin, 0.40 for albumin (Reichl et al., 1982b), while the ratios for apoA-I or apoB are between 0.12-0.14 and 0.06-0.09 respectively (Reichl and Pflug, 1982a, 1985, Sloop et al., 1983a). Lymph contains relatively higher levels of apoE and apoA-IV that are associated with particles larger than $HDL_2$ (Sloop et al., 1983a, Dory et al., 1985, Reichl et al., 1985).

Although the lymph/plasma ratio of total cholesterol varied within animal species and tissues (Forte et al., 1983, Julien et al., 1981, 1984, Reichl et al., 1985), a higher UC/CE ratio is always found in interstitial fluid or lymph compared to plasma (Sloop et al., 1983a, Forte et al., 1983, Dory et al., 1985, Julien et al., 1988, Rudra et al., 1984), which is consistent with the low LCAT activity in interstitial fluid/lymph (Julien et al., 1988). Free cholesterol of interstitial fluid/lymph appears mainly in HDL fraction (Sloop et al., 1983a, Dory et al., 1985, Forte et al., 1983, Julien et al., 1984), and cholesterol-feeding can particularly enhance HDL free cholesterol (Sloop et al., 1983a, Dory et al., 1985) and the apoA-I, apoA-IV and apoE levels (Sloop et al., 1983a, b, Julien et al.,

1984, 1988). Moreover, human lymph contains high level of SM with an average lymph/plasma ratio of 0.18, in contrast to that of 0.05 for PC (Reichl et al., 1992).

3.2.2. Size and morphology of extravascular HDL

Lymph HDL shows a broader range of size distribution than plasma HDL with an average size bigger than that of plasma (Lefevre et al., 1988, Reichl and Pflug, 1982a, Sloop et al., 1983a, Dory et al., 1985, 1986, Reichl et al., 1985). Small spheres, large spheres, as well as large discs of HDL have all been identified from the d=1.063-1.21 g/ml fraction of lymph, where discoidal HDL accounts for a higher proportion than that of plasma HDL (Sloop et al., 1983a, b, Forte et al., 1983, Reichl et al., 1985, Dory et al., 1985). The ratio of pre β/α HDL concentration, especially the pre $\beta_1$ and pre α HDL subpopulations (Asztalos et al., 1993b) is higher than that of plasma (Reichl et al., 1991, Lefevre et al., 1988, Dory et al., 1985). Cholesterol-feeding also results in a significant increase of pre β-HDL in plasma (Lefevre et al., 1988) and larger discoidal HDL in lymph (Sloop et al., 1983a, Dory et al., 1985), but a reduced pre β-HDL level in lymph (Lefevre et al., 1988).

### *3.3 Reconstitution of HDL-like Particles*

Event though HDL has been classified into many different subspecies as described above, these subspecies of HDL are still heterogeneous because of the

presence of variable amounts of minor protein components and the diversity of their lipid constituents. Thus, attempts to obtain detailed molecular information on this lipoprotein have been hindered by their heterogeneity. In recent years, HDL-like particles, reconstituted by assembling the basic components of native HDL *in vitro,* have been employed to facilitate the structure/function studies of HDL.

Three methods have been applied to the co-dispersion and interaction of apolipoproteins with selected synthetic or native phosphatidylcholine (PC) having different fatty-acyl chains: i) spontaneous interaction of apolipoproteins with lipid vesicles; ii) detergent-mediated reconstitution of these components; and iii) co-sonication of these components (review by Jonas, 1986). In addition, some laboratories have produced spherical reconstituted LpA-I by incubating reconstituted discoidal LpA-I particles with LDL and LCAT (Jonas et al., 1990, 1994, Rye and Barter, 1994, Gong et al., 1988).

ApoA-I contains amphipathic α-helical structures that have an affinity to lipids (Brewer et al., 1972, Segrest et al., 1974) (see 5.2.4 for detailed information on the structure and function of this apolipoprotein). Previous investigations have shown that apoA-I could spontaneously associate with dimyristoryl phosphatidylcholine (DMPC) and cholesterol in a transition temperature sensitive manner, and the sizes of reconstituted LpA-I were positively related to the initial ratio of PC/apoA-I (Antonov et al., 1985). This reconstitution method is limited

since the homogeneity and composition of the reconstituted particles cannot be well controlled.

The detergent-mediated reconstitution method, in which PL is dispersed with sodium cholate, allows the incorporation of a higher level of PL with an apolipoprotein. In this method, dried phospholipids are first made into very large multilamellar vesicles (MLV), which are then dispersed with sodium cholate to form mixed micelles of lipids and detergent, to which the apolipoprotein is incorporated. The physico-chemical properties of the reconstituted particles are determined by the fatty acyl chains of PC, the types of apolipoproteins, the PC/ apolipoprotein ratio, as well as the presence or absence of cholesterol (Zorich et al., 1987, Sparks et al., 1992a, b, 1993, Nichols et al., 1983). Usually, with the increase of PC/apoA-I molar ratio in the initial reconstitution mixture, the components are assembled into larger discoidal particles with corresponding reduction in the smaller subclasses (Nichols et al., 1983). The HDL-like particles made by dispersing phospholipid with cholate anions are usually heterogeneous when prepared at 4°C (Jonas et al., 1986) but homogenous at 37°C (Sparks et al., 1992a, b).

It was in the early seventies that studies by Hirz and Scanu (1970) showed that spherical lipoprotein particles containing either apoA-I or apoA-II could be reconstituted by co-sonication of purified lipids and apolipoproteins. This

discovery is important since the spherical shape of these reconstituted lipoproteins is compatible to the majority of native HDL particles; unfortunately however, size characterization showed that these preparations were as heterogeneous as native HDL. Therefore, this method has recently been modified by Sparks and colleagues (1992b, 1995) so that highly homogeneous spherical LpA-I and LpA-I /A-II could be produced. Reconstituted HDL-like particles prepared by sonication method are usually smaller than that prepared by detergent-dispersion method, and the incorporation of phospholipids into apolipoprotein is less efficient.

## 4. Mechanism of Cholesterol Efflux from Extrahepatic Cells

The mechanism of cholesterol efflux from peripheral cells is still unclear. At present, two main pathways have been proposed, which are the aqueous diffusion efflux of UC from cell plasma membrane, and the HDL receptor mediated translocation-dependent efflux of cholesterol. In addition, a pathway of HDL particle retroendocytosis occurring mainly in hepatoma cells and macrophages has been suggested as a supplementary pathway to cellular cholesterol efflux. The quantitative importance of this process in reverse cholesterol transport remains to be established.

### *4.1. Aqueous Diffusion Efflux of Cholesterol from Extrahepatic Cells*

The diffusion process of cellular cholesterol efflux through an aqueous phase surrounding the cells depends on a cholesterol concentration gradient between the donor and the acceptor; however, an intermediate step is involved. Two general types of intermediates, a transient collision complex and a water-soluble lipid monomer, have been postulated. In transient collision model, extracellular acceptors collide with plasma membrane, and then the cholesterol-acceptor complexes are released from the plasma membrane; while in the water-soluble lipid monomer model, cholesterol is first desorbed from plasma membrane into the aqueous phase as a monomer, then collides with extracellular cholesterol acceptors (Phillips et al., 1987).

In addition, a three step activation-collision model has been suggested recently, where cholesterol or phospholipid molecules in plasma membrane are first activated, then acceptors collide with and bind to the activated molecules to form intermediates which are then dissipated instantly from plasma membrane (Steck et al., 1988). Under the condition that the collision is not a rate-limiting factor, e.g. at a high concentration of acceptors, the overall process of activation-collision would appear as a simple first-order process which is kinetically indistinguishable from the aqueous diffusion mechanism.

It appears that aqueous diffusional efflux of cholesterol is independent from the binding ability of HDL to plasma membrane. As has been reported, there was no relationship between the UC efflux rate and the binding ability of native HDL to cell plasma membrane (Mendel and Kunitake, 1988, Johnson et al., 1988). Similarly, tetranitromethane (TNM) or dimethylsuberimidate (DMS) treatment reduced the ability of HDL to bind to cell plasma membrane (Chacko, 1985, Chacko et al., 1988, 1987, Schouten et al., 1988); however, not the ability to release cellular cholesterol (Slotte et al., 1987, Karlin et al., 1987, Mahlberg et al., 1991). LpA-I reconstituted with mutant apoA-I $pro^{165}$-->Arg showed significantly decreased capability to promote cholesterol efflux; however, the membrane binding ability of the LpA-I to adipocytes was similar to that of native HDL, or to the LpA-I reconstituted with other types of mutant apoA-I that did not affect their abilities to release cellular UC (von Eckardstein et al., 1993). Moreover, although LpA-I and LpA-I/A-II had similar abilities to bind to plasma membrane of adipocytes, only LpA-I was able to promote the efflux of cholesterol from these cells (Barbaras et al., 1987a, b, 1988, Barkia et al., 1988, 1991). This postulate is further supported by the fact that HDL could induce the efflux of cholesterol from isolated bovine heart mitochondria which lacked lipoprotein receptors (van Hensden et al., 1989, Cabezas et al., 1993).

### *4.2. HDL Receptor Mediated Cholesterol Efflux from Cells*

Studies from many different laboratories have identified a number of HDL binding candidate proteins from the plasma membrane of different cells by ligand blot method; however, the molecular weights of these potential receptors are quite different (80 - 130 kDa). Among them, a 110 kDa glycoprotein is the one commonly identified in peripheral cells (Biesbroeck et al., 1983, Graham and Oram, 1987, Hokland et al., 1992, Oram et al., 1991). However, the HDL binding sites in many aspects cannot satisfy the criteria of a classical biological receptor in plasma membranes although it appears that these proteins are found mainly in the 5'-nucleotidase rich-subcellular fraction (Hokland et al., 1992), and the mass and the binding activities of these sites can be regulated by cellular cholesterol content (Schmitz et al., 1985, Oram et al., 1983, 1987, Brinton et al., 1985, Graham and Oram, 1987, Bernini et al., 1991, Oppenheimer et al., 1987, Tozuka and Fidge, 1989). For example, a novel 150 kDa protein encoded by a cDNA clone screened from the monoclonal antibody against the 110 kDa protein was found mainly in cytosol fractions, and the predicted structure of this protein does not conform to that of any known receptor (McKnight et al., 1992). Furthermore, the binding sites of HDL exhibit low specificity that bind not only to native HDL, reconstituted LpA-I, but also to reconstituted LpA-II and LpC-III (Brinton et al., 1984, Rifici

and Eder, 1984, Fidge and Nestel, 1985, Graham and Oram, 1987, Mitchel et al., 1987, Fong et al., 1987, Vadiveloo and Fidge, 1992).

Nevertheless, studies have suggested that the binding of HDL to cell plasma membrane receptors is the necessary step for the translocation of cholesterol from intracellular pools to cell surface. The decrease of HDL binding sites in differentiated aortic smooth muscle cells was associated with a reduction of intracellular cholesterol efflux (Dusserre et al., 1994). TNM-treated HDL lost its ability to bind to cell plasma membrane as well as the ability to stimulate the translocation of intracellular cholesterol (Oram et al., 1991, Aviram et al., 1989, Slotte et al., 1987, Brinton et al., 1986). The translocation of cholesterol appeared to cause the depletion of sterol from microsomal pool since exposure of cells to $HDL_3$ led to a time-dependent decrease of ACAT activity (Brinton et al., 1986). However, the progressive decrease in the ratios of membrane/lysosomal cholesterol efflux has been observed following the elongation of incubation time, which suggested a mobilization of cholesterol from lysosomes (Mahlberg et al., 1991). It is apolipoprotein that has been proposed to serve as the ligand between HDL and the plasma membrane because treatment of $HDL_3$ with trypsin and pronase could reduce the removal of sterol from intracellular pool, but not from the plasma membrane (Oram et al., 1991, Herzyk et al., 1988).

Membrane signaling systems have been reported to function in the process of cellular cholesterol efflux. A transient increase of diacylglycerol (DAG) (Mendez et al., 1990, Walter et al., 1995), inositol triphosphate ($IP_3$) (Möllers et al., 1995), as well as the subsequent mobilization of intracellular calcium (Möllers et al., 1995) and the activation of plasma membrane protein kinase C (PKC) have been observed when $HDL_3$ or reconstituted apoA-I/DMPC complexes were incubated with cholesterol-loaded fibroblasts (Walter et al., 1995, Oram et al., 1991, Mendez et al., 1991a, b, 1990) or with cholesterol-loaded ob-1771 adipocytes (Theret et al., 1990). In addition, the function of HDL in promoting intracellular sterol translocation could be simulated by PKC activators, such as phorbol myristic acetate (PMA), 1,2-dioctanoyl-glycerol or calcium ionophore A 23187 (Mendez et al 1989, 1990, 1991a, b, Duverger et al., 1990), and be interfered by PKC inhibitor such as sphingosine (Mendez et al., 1989). Protein-receptor interaction is the necessary step in activating cellular signaling system. As long as HDL loses its ability to bind to cell plasma membrane, such as with TNM treatment, it can neither enhance the level of DAG nor stimulate the activity of PKC (Mendez et al., 1990, 1991a, Walter et al., 1995). In addition, the function of HDL in cellular signaling pathway cannot be mimicked by liposomes (Walter et al., 1995). The PKC mediated cholesterol efflux occurs mainly in lipid-loaded

cells, such as adipocytes or foam cells. Peripheral cells, when not loaded with cholesterol, show little or no efflux via this pathway (Oram et al., 1991).

Different from the PKC-mediated signaling pathway, cAMP and protein kinase A (PKA) are neither involved in the hydrolysis of CE in J774 macrophages (Bernard et al., 1991) nor in HDL stimulated cholesterol efflux from human skin fibroblasts and bovine aortic endothelial cells. However, cAMP analogues, phosphodiesterase inhibitor, and adenyl cyclase activator have been reported to stimulate the cholesterol efflux process (Hokland et al., 1993).

It is quite possible that the aqueous diffusion- and the translocation-pathways exist concomitantly and reflect the different aspect of the efflux process. The aqueous diffusion process may be predominant during the initial stage of efflux, since the desorption of cholesterol from plasma membrane is very rapid whether directly from the outer leaflet of plasma membrane or from the inner leaflet by the translocation of UC via flip-flop movement. In contrast, the translocation of intracellular UC to plasma membrane is relatively slow. Following the elongation of incubation time, the cholesterol translocated from intracellular pools to plasma membrane will increase progressively. Since CE is the major storage form of cholesterol in cholesterol-loaded cells and foam cells, the hydrolysis, translocation and removal of intracellular CE have been postulated to play an important role in the regression of atherosclerosis.

### *4.3. Retroendocytosis of HDL Particles*

As previously mentioned, an additional pathway involving retroendocytosis of HDL has been suggested to be involved in cellular cholesterol efflux in hepatoma and macrophages. In this reversible pathway, receptor binding leads to the internalization of HDL and the direct interaction of HDL with intracellular lipid droplets. Different from LDL particles which undergo intracellular degradation upon uptake by cells, HDL will bind intracellular cholesterol molecules, recycle to the cell surface, and then release them into extracellular fluid.

HDL retroendocytosis could be demonstrated directly by the presence of HDL conjugated with colloidal gold particles (Schmitz et al., 1985, 1988), fluorescein isothiocyanate (Rahim et al., 1991), ferritin (Takahashi et al., 1989) or horseradish peroxidase (Takahashi et al., 1989) inside rat peritoneal macrophages. Electronmicroscopy of human monocytes-derived macrophages (Alam et al., 1989) and rat hepatoma Fu5AH cells (DeLamatre et al., 1990) has showed that HDL were endocytosed, delivered to the endosomal or trans-Golgi compartment to be re-packaged into secretary vesicles, then to be released as larger $HDL_2$-like particles containing apoE. Therefore, retroendocytosis may produce HDL particles that are capable of binding to apoB/E receptors, thus providing an alternative

the formation of foam cells. In the monocytes of patients with Tangier disease, the defective HDL retroendocytosis results in the accumulation of HDL in lysosomes for degradation, which may contribute to the low plasma HDL level and the abnormal storage of lipids in these patients (Schmitz et al., 1985).

## 5. Factors Involved in HDL-Induced Cellular Cholesterol Efflux

The mechanism of HDL induced cellular cholesterol efflux has been investigated in cultured cells by numerous laboratories using both native HDL and reconstituted HDL-like particles as acceptors. The review of studies of nearly three decades on cholesterol efflux shows that the ability of HDL to stimulate cholesterol efflux depends greatly on the specificity of cell species and the inherent properties of different HDL subspecies. Sometimes, certain plasma enzymes may be involved indirectly in this process by affecting the conversion/remodeling of HDL particles.

### *5.1. Cell Specificity of Cellular Cholesterol Efflux*

The effects of HDL particles on cellular cholesterol metabolism depend greatly on individual cell population. Data summarized in a review by Rothblat and Phillips (1986) showed that the T1/2 of cholesterol efflux from different types of cells to a given acceptor varied greatly. Usually, hepatocyte-derived cells such

of cells to a given acceptor varied greatly. Usually, hepatocyte-derived cells such as Fu5AH and HepG2 have the fastest rate, followed by epithelial L cells, mouse J774 macrophages, and then fibroblasts. HDL has a higher ability to release cellular cholesterol from bovine vascular endothelial cells than that from vascular smooth muscle cells (Savion and Kotev-Emth, 1989). Similarly, a slow release of cellular cholesterol from aortic smooth muscle cells to lipid-free apoA-I compared to human skin fibroblasts and mouse peritoneal macrophages has also been observed (Komaba et al., 1992, Li and Yokoyama, 1995, Li et al., 1993). In addition, different cell species have different specificity towards a given HDL subspecies for the release of cholesterol. For example; egg yolk phosphatidylcholine (EYPC)/apoA-I complex has a higher ability than EYPC/apoA-II or EYPC/Cs complex to release cholesterol from J774 macrophages; however, the efflux rates of cellular cholesterol from Fu5AH rat hepatoma or rabbit aortic smooth muscle cells to these complexes were basically the same (Mahlberg and Rothblat, 1992).

The mechanism for the distinct rates of cellular cholesterol efflux from specific types of cells is still unclear. Cellular cholesterol content may be one of the factors involved since increase of cellular cholesterol content results in corresponding enhancement of cholesterol efflux to HDL (Johnson et al., 1988, Mendel et al., 1988). However, in contrast to this point of view, a study has shown

that cholesterol efflux from intact cells was similar to that from plasma membrane vesicles isolated from the same cells, which suggests the importance of the intrinsic property of plasma membrane in this process (Bellini et al., 1984).

It is generally believed that the phospholipid composition of a cell plasma membrane determines the association of cholesterol with that particular compartment. Among the phospholipid species present in cell plasma membrane, SM has been reported to have the highest affinity to cholesterol in small unilamellar vesicles (Lund-Katz et al., 1988, Yeagle and Young, 1986, Fugler et al., 1985). The co-distribution of cellular cholesterol and SM has been observed (Lange et al., 1989, van Blittersuijk et al., 1987) although they are on the different leaflets of the membrane bilayer (Schroeder et al., 1991). Incorporation of exogenous SM into cultured fibroblasts results in a marked increase of cholesterol biosynthesis and reduction of LDL binding and degradation (Gatt and Bierman, 1980). In contrast, depletion of SM from plasma membrane of fibroblasts by exogenous neutral sphingomyelinase (SMase) led to a rapid flow of cholesterol from cell surface into intracellular pools (Slotte et al., 1989, 1990, Pörn et al., 1991, Pörn and Slotte, 1990) as indicated by the increase of ACAT activity, the subsequent enhanced esterification of plasma membrane-derived free $^{3}$H-cholesterol, and the increase of cellular CE mass (Slotte and Bierman, 1988, Pörn and Slotte, 1990). As well, cholesterol de novo synthesis is down-regulated (Slotte

and Bierman, 1988, Gupta and Rudney, 1991). The alteration of cellular SM level will produce a profound effect on cellular cholesterol efflux. As expected, the degradation of plasma membrane SM with exogenous SMase led to a significant increase of cellular cholesterol efflux from human skin fibroblasts (Pörn et al., 1993).

PC seems not critical for the rate of cellular cholesterol efflux, although it is the predominant phospholipid in cell plasma membrane. It has been reported that degradation of approximately 15% of PC from cell plasma membrane by a PC specific phospholipase C affected neither the distribution nor the efflux rate of cellular cholesterol in fibroblasts (Pörn et al., 1993). However, the fatty acid profile of cell plasma membrane has been reported to influence cholesterol efflux rate. Cholesterol efflux was increased significantly upon incubation of human endothelial cells with palmitic acid, but not in the incubations with oleic, linoleic, arachidonic and eicosapentaenoic acids (Kilsdonk et al., 1992).

### *5.2. HDL Specificity of Cellular Cholesterol Efflux*

HDL is a highly heterogeneous lipoprotein population in both plasma and interstitial fluid/lymph with large variations in composition and size. Studies have demonstrated that the efflux rates of cellular cholesterol from given cells depend on the inherent characteristics of HDL, which can be summarized as follows:

5.2.1. The effects of surface lipid composition of HDL on cholesterol efflux

HDL has been shown to contain three main subclasses of phospholipid, where PC, SM, and phosphatidylinositol (PI) count for 86±4%, 10±3% and 4±0% respectively of its total phospholipid mass. Lysolecithin, phosphatidylserine (PS) and phosphatidylethanolamine (PE) are present only in trace amount (Skipski, 1972).

The role of PC in HDL-mediated cholesterol homeostasis has been emphasized since it is the major phospholipid component of HDL, and a specific decrease of this phospholipid has been reported in hyperlipidemia (Kunz et al., 1994). PC binds cholesterol selectively with a high affinity (Lund-Katz et al., 1988, Yeagle and Young, 1986). When the PC content of HDL is reduced by treatment with phospholipase $A_2$, the ability of the treated HDL to release cholesterol from cells is significantly reduced (Johnson et al., 1986). Meanwhile, the influx of cholesterol from the modified HDL to cells is increased (Collet et al., 1988). In addition, phospholipid liposomes by themselves were also able to promote dose-dependent efflux of cholesterol from human GM3468 fibroblasts (DeLamatre et al., 1986, van Heusden et al., 1989), although the addition of apolipoproteins made the PC vesicles turn into much more effective acceptors (Stein et al., 1986, van Heusden et al., 1989, DeLamatre et al., 1986). The increased efficiency should be attributed at least to the ability of apolipoproteins to

convert liposomes into small apolipoprotein-phospholipid complexes, thereby increasing the relative surface area of these particles to accept the cell-derived cholesterol (DeLamatre et al., 1986).

SM has been postulated to enhance the ability of HDL to release cellular cholesterol since this phospholipid has a high affinity for cholesterol. As expected, a significant positive correlation between the SM liposome concentrations added into the culture media and the rates of cellular cholesterol efflux has been reported (Stein et al., 1988, 1989). The relatively high ratio of SM in pre $\beta_1$-HDL (Fielding and Fielding, 1995a) may also be the factor that turns this species of LpA-I particles into an avid cholesterol acceptor (Castro and Fielding, 1988).

It has been demonstrated that PI contributed partially to the negative surface charge of HDL (Davidson et al., 1994a); however, the function of this phospholipid in the ability of HDL to release cholesterol from peripheral cells has not been reported. Phospholipid mixtures made from PE and apoA-I, apoA-IV, apoE, or apoC showed low efficiencies in releasing cellular cholesterol from fibroblasts compared to PC/apolipoprotein mixtures (Stein et al., 1986).

The concentration gradient of cholesterol, usually represented by UC/PL ratio between cell plasma membrane and HDL, determines the direction of cholesterol flux. Since this ratio in the cell plasma membrane of a given cell species is relatively constant, the UC/PL ratio of HDL is, therefore, the critical

factor in determining HDL either a donor or an acceptor of cholesterol. It has been reported that reconstituted LpA-I containing free cholesterol had a reduced ability for cholesterol efflux from fibroblasts compared to the LpA-I without cholesterol (Agnani and Marcel, 1993).

5.2.2. The effects of HDL apolipoprotein composition on cholesterol efflux.

As mentioned above, all apolipoproteins except apoB have been identified in HDL, and the roles of HDL apolipoproteins on cellular cholesterol efflux have been widely studied either through the removal of lipoproteins containing specific apolipoproteins from HDL by immunoaffinity chromatography, or through the assembly of phospholipids with different apolipoproteins into reconstituted HDL-like particles.

The importance of apoA-I-containing lipoproteins in cholesterol efflux has been universally demonstrated. A pioneer study showed that the removal of apoA-I from plasma resulted in significant reduction of cholesterol efflux compared to that of normal plasma (Fielding and Moser, 1982). In selected cell lines, such as normal human skin fibroblasts, FuA5H rat hepatoma cells, rabbit aortic smooth muscle cells, mouse macrophages, bovine aortic endothelial cells and bovine aortic smooth muscle cells, both native LpA-I and LpA-I/A-II were able to promote cholesterol efflux, but LpA-I was more efficient than LpA-I/A-II (Johnson et al., 1991, von Hodenberg et al., 1991, de la Llera Moya et al., 1994, Oikawa et al.,

1993, Miida et al., 1990, Stein et al., 1995, Ohta et al., 1992, Huang et al., 1995). $HDL_3$ whose apoA-I contents have been progressively replaced by apoA-II showed a corresponding decrease in their abilities to release cellular cholesterol from Fu5AH cells (Lagrost et al., 1995). Similar results have also been observed with mouse macrophages, but not with aortic smooth muscle cells (Stein et al., 1995). In cholesterol-loaded ob-1771 mouse adipocytes, LpA-I rather than LpA-I/A-II was able to promote cholesterol efflux, (Barbaras et al., 1986, 1987a, b, 1988, Barkia et al., 1988, 1991), and in some cases LpA-I/A-II even showed an antagonistic effect on the cholesterol efflux induced by LpA-I (Barkia et al., 1991).

The importance of apoA-I containing lipoproteins in cellular cholesterol efflux is also confirmed by studies using reconstituted HDL-like particles. Lipoproteins containing apoA-IV, apoE, or apoC were all active in removing cellular cholesterol; however, their efficiencies in this process depended on cell species (Mahlberg and Rothblat, 1992, Mahlberg et al., 1991, DeLamatre et al., 1986, Bellini et al., 1984, Steinmetz et al., 1990). ApoC-containing liposomes were less efficient than apoA-I, apoA-IV or apoE containing liposomes in human skin fibroblasts (Stein et al., 1986).

5.2.3. Plasma HDL subspecies as initial acceptors of cell-derived cholesterol

Three subspecies of HDL have been reported as the first acceptors for cell-derived cholesterol when fresh plasma is incubated with human skin fibroblasts for a short period of time. They are the pre $\beta_1$-HDL that contains only apoA-I and exhibits a pre β-migration upon electrophoresis (Castro and Fielding, 1988), the γ–LpE that contains only apoE and exhibits a γ electrophoretic mobility (Huang et al., 1994), and the LpA-IV that contains only apoA-IV and displays a "slow α-mobility" (von Eckardstein et al., 1995). However, only the subsequent transfer from pre $\beta_1$-HDL has been studied extensively, where the cell-derived cholesterol is transferred from a pre $\beta_1$-LpA-I to a pre $\beta_2$,-LpA-I within 1 min, then to pre $\beta_3$-LpA-I and eventually to an α–HDL within 15 min (Castro and Fielding, 1988, Francone and Fielding, 1990b). In contrast to the LDL-derived cholesterol that is directly transferred to and esterified in α-HDL, the cell-derived cholesterol transferred to pre $\beta_1$-LpA-I is esterified in both pre $\beta_3$-LpA-I and α-HDL (Francone and Fielding, 1990b, Miida et al., 1990, Huang et al., 1993). The pre β-dependent efflux of cellular cholesterol appears to contribute a major portion of cellular cholesterol efflux, since depletion of 75 % plasma pre β-HDL by incubation of fresh plasma at 37°C for 1.5-2 h (Miida et al., 1992, Kunitake et al., 1992) reduced cholesterol efflux by up to 58% (Kawano et al., 1993). The pre β-

HDL-dependent cholesterol efflux is also protease-sensitive and linked to LCAT mediated esterification (Fielding and Fielding, 1995a, Castro and Fielding, 1988), and seems completely absent in erythrocytes (Kawano et al., 1993). Usually, only approximately 5-10% of cell-derived cholesterol is transferred as CE from HDL to LDL and VLDL, and the rest goes directly to LDL as UC (Francone et al., 1989, 1990a, Huang et al., 1993).

Up to now, the origin of pre β HDL particles has not been fully understood. Studies have suggested that this lipoprotein subspecies can be produced as a result of the transformation of $HDL_2$, $HDL_3$, and reconstituted HDL-like particles by the action of LCAT (Neary et al., 1991), and the hydrolysis of triglyceride-rich by hepatic triacylglyceride lipase (HTGL) (Barrans et al., 1994, Clay et al., 1991), CETP (Barrans et al., 1994, Clay et al., 1992, Newnham et al., 1992, Hennessy et al, 1993, Rye et al., 1995, Liang et al., 1994) and phospholipid transfer protein (PLTP) (Tu et al., 1993, Jauhiainen et al., 1993, Albers et al., 1995). In recent years, an extracellular assembly process of pre β-LpA-I by transfer of cholesterol and phospholipids from extrahepatic cells to lipid-free apolipoproteins has been suggested (Cazarnecka and Yokoyama, 1995, Hare and Yokoyama 1991, 1992, Bielicki et al., 1991, 1992, Huang et al., 1995). The pre β-LpA-I can be the precursor of α-HDL since the sequential appearance of larger complexes as a

function of time can be observed in the incubation medium of lipid-free apoA-I with non-transfected CHO-C19 cells (Forte et al., 1993). The conversion of pre β-LpA-I to α-HDL has also been demonstrated by *in vitro* incubation of pre β-LpA-I with fresh plasma (Huang et al., 1995), or by direct incubation of fresh plasma at $37^0$C for 1-2 hr. (Miida et al., 1992, Kunitake et al., 1992, Neary et al., 1991). Therefore, this subspecies of HDL may exist *in vivo* as a transient population of particles which can be formed and converted constantly (Fig. 6).

5.2.4. The structure of apoA-I in LpA-I in relation to membrane binding and cellular cholesterol efflux

ApoA-I is a major protein constituent of HDL known to be secreted as proapoA-I (a single polypeptide of 249 amino acids) from the liver (Zannis et al., 1982) and the small intestine (Zannis et al., 1982, 1980), and undergoes an extracellular transformation to apoA-I (243 aa) (Edelstein et al., 1983, Ghiselli et al., 1983, Bojanovski et al., 1985). Like all other exchangeable apolipoproteins, a large portion of the protein is formed by multiple 22 mer tandem repeats that each generally consists of a 17 mer segment of amphipathic α-helix and an extended beta-strand of 5 residues for the most part centered around α-helix-breaking proline residues (Boguski et al., 1986, Segrest et al., 1990). These amphipathic α-helix structures, usually with their charged residues facing the aqueous medium

and non-charged residues facing the acyl chains of the phospholipid bilayer, are the domains of apoA-I to interact with lipids (Segrest et al., 1992). Lipid association could further increase the α-helicity of the peptides (Kanellis et al., 1980, Anantharamaiah et al., 1988), and changed the overall structure of apoA-I from a soluble globular conformation to the lipid-bound conformation on the surface of lipoprotein (Calabresi et al., 1993). It has been reported that one 22 mer repeat can be the basic unit to interact with lipid and to form discoidal particles; however, the presence of two antiparallel 22 mer repeats significantly increases the stability of the corresponding LpA-I (Vanloo et al., 1995). This evidence suggests strongly that the 44 mers represent in fact the minimum functional domain of apoA-I in LpA-I. It has been demonstrated that the conformation of apoA-I in HDL may be influenced by other components of HDL such as phospholipid and cholesterol (Collet et al., 1991, Calabresi et al., 1993, Bergeron et al., 1995, Sparks et al., 1992a, b, 1993, Jonas et al., 1990). The increase of phospholipid content of reconstituted LpA-I particles contributed to a greater apoA-I stability (Sparks et al., 1992a, b), which presumably reflects the increased interactions of amphipathic helices with the lipids.

It has been known that epitope expression of apoA-I in HDL affects the function of HDL to bind to cell plasma membrane (Leblond and Marcel, 1991) and to interact with LCAT (Meng et al., 1993, Banka et al., 1991). Studies have been

conducted to search for the relationship between the expression of apoA-I epitopes and the function of HDL to release cellular cholesterol. Although the results vary from laboratory to laboratory, the effective epitopes are located basically within the two central lipid binding fragments (amino acid residues 66-120, and 147-185) described by Segrest et al (1992). For example, LpA-I particles reconstituted with a mutant apoA-I ($Pro^{165}$-Arg) were defective in releasing cellular cholesterol from adipocytes and peritoneal macrophages compared to LpA-I made of wide-type apoA-I (von Eckardstein et al., 1993). Monoclonal antibodies (mAb) reacting with amino acid residues 96-111 and 74-105 of apoA-I blocked the release of cholesterol from THP-1 monocytic cells to native HDL or proteioliposomes (Banka et al., 1994). Similar inhibitory effect on cellular efflux was also observed with mAb A44 which binds to amino acid residues of 149-186 (Luchoomun et al., 1994). Recently, it was found that a mAb binding to an epitope constituted by the sequence LQEKLSPL (137-144) and expressed exclusively in pre $\beta_1$-HDL could reduce the cholesterol efflux by 63% (Fielding et al., 1994). In summary, the inconsistent results as mentioned above might indicate that no specific epitope but the lipid binding affinity can be linked with the function of HDL to release cellular cholesterol. This conclusion is further supported by the fact that synthetic class A $\alpha$-helical amphipathic peptides which have no homology with apoA-I are also able

to remove cellular cholesterol (Davidson et al., 1994b, Mendez et al., 1994, Yancey et al., 1995).

### *5.3. Effects of LCAT on Cholesterol Efflux*

The function of LCAT is to transfer an acyl chain from the sn-2 position of PC to cholesterol, thus produce lyso-PCs and cholesterol ester. As previously mentioned, cholesterol esterification catalysed by LCAT contributes to the maturation of HDL, where nascent discoidal HDL is converted into mature spherical HDL (Vanloo et al., 1992). Theoretically, the movement of CE away from HDL surface helps maintain a constant concentration gradient of cholesterol between the cell plasma membrane and the surface of HDL, thus favors the net release of cellular cholesterol. However, studies of native HDL with cholesterol loaded EA.hy 926 human endothelial cells (Kilsdonk et al., 1993a), and of free apoA-I or pre β–HDL with cholesterol loaded macrophages and human skin fibroblasts (Cazarnecka and Yokoyama, 1995) have suggested that LCAT induced cholesterol esterification on HDL is unable to enhance cellular cholesterol efflux. In contrast, the influx of cholesterol from HDL to cells can be reduced by LCAT, thus a net cholesterol efflux was formed (Cazarnecka and Yokoyama, 1995). A recent study has suggested that the phenotype observed in LCAT deficiency is directly related to the function of HDL on cellular cholesterol efflux. A 50%

reduction in the capacity to accept cell-derived cholesterol was observed in the incubation of macrophage foam cells with plasma LpA-I or LpA-I/A-II particles from homozygous familial LCAT deficiency patients; however, a 20% increase was observed with lipoproteins from heterozygous familial LCAT deficiency patients (Ohta et al., 1994b).

LCAT may also regulate RCT indirectly by modifying the size of HDL, such as for the conversion of pre β–HDL into α-HDL (Miida et al., 1992, Kunitake et al., 1992, Neary et al., 1991), and for the formation of larger and smaller HDL particles upon incubation of plasma at $37^0$C (Rajaram and Barter, 1986, Cheung and Wolf, 1989, Nichols et al., 1989).

## 6. Structure of Albumin and Its Role in Cholesterol Transport

Albumin is the most abundant protein in plasma, which accounts for 60% of total protein in human plasma with a typical concentration of 50 mg/ml (Peters, 1975). This protein is folded in a cylindrical structure formed by six antiparallel hydrophobic helical segments, and therefore it is able to interact with hydrophobic molecules. The three main functions that have been attributed to albumin are to maintain blood pH and the blood osmotic pressure, and to bind to a variety of ligands. The most predominant ligand of albumin is fatty acid which is otherwise insoluble in circulating plasma. Although each albumin molecule is able to bind up

to six molecules of fatty acids (reviewed by Cater and He, 1990, Spector, 1986), this protein normally carries only 0.5 - 2 fatty-acid molecules in plasma, which turn over actively with a half-life of 1- 2 min (Spector, 1986). All the fatty acids including long-, medium- and short-chain in triacylglycerols are able to bind to albumin (Wang et al., 1993). In addition, albumin is also able to bind to bile acids (Brock, 1976, Roda et al., 1982), steroid hormones and some hydrophobic drugs, such as digitoxin (Brock, 1976).

Previous studies have indicated the possible involvement of albumin in cholesterol metabolism and in atherogenesis. Epidemiological studies have identified a positive correlation between the ratios of total cholesterol/albumin and total cholesterol/HDL ($r=0.89$, $p<0.001$) (Nanji, 1983). Moreover, relatively low serum albumin has been suggested as a predictor of increased incidence and mortality of CAD (Gillum and Makuc, 1992, Gillum, 1993). Albumin may also play a role in the transport of lipid and cholesterol in plasma. For example, removal of albumin from plasma by affinity chromatography results in more than 50% decrease of cholesterol efflux from human skin fibroblasts compared to normal plasma (Fielding and Moser, 1982). A concentration dependent stimulation of cholesterol efflux from the same cells by BSA has been reported by Mendel and Kunitake (1988). Moreover, incubation of BSA with cholesterol-enriched Fu5AH

at hepatoma or human skin fibroblasts at a concentration of 100 μg/ml for 8 h reduces cellular cholesterol content up to 32% (Johnson et al., 1991).

The importance of albumin in lipid metabolism is clearly demonstrated by the occurrence of lipid metabolic disorders accompanying with the disorders of albumin metabolism, such as in patients with analbuminemia, with nephrotic syndrome, as well as in Nagase analbuminemic rats (NAR). Although no important clinical symptoms showed, analbumin and nephrotic syndrome patients have an altered protein and lipid metabolism such as hyperlipidemia, hypercholesterolemia and hypertriglyceridemia (Cohen, 1980, Baldo-Enzi et al., 1987). The changes included the increases of apoB and LDL, slight increases of apoA-I in total serum and in $HDL_3$ fraction, and a decrease of apoA-I in the $HDL_2$ fraction (Baldo-Enzi et al., 1987). Enhanced plasma triacylglycerol, phospholipid, total cholesterol as well as apolipoproteins have been observed in NAR (Nagase et al., 1979, Kikuchi et al., 1983, Takahaski et al., 1983, Van Tol et al., 1991, Catanozzi et al., 1994).

Contrary to the above evidence, analbuminemic individuals or analbuminemic rats do not show any increased risk for cardiovascular disease in spite of the extensive alteration on lipid metabolism (Baldo-Enzi et al., 1987, Joles et al., 1991). Whether the enhanced apoA-I in $HDL_3$ fraction functions as a supplement factor in the anti-atherogentic process remains unknown. Nevertheless,

the above studies have strongly suggested a relationship between the function of albumin and cholesterol transport.

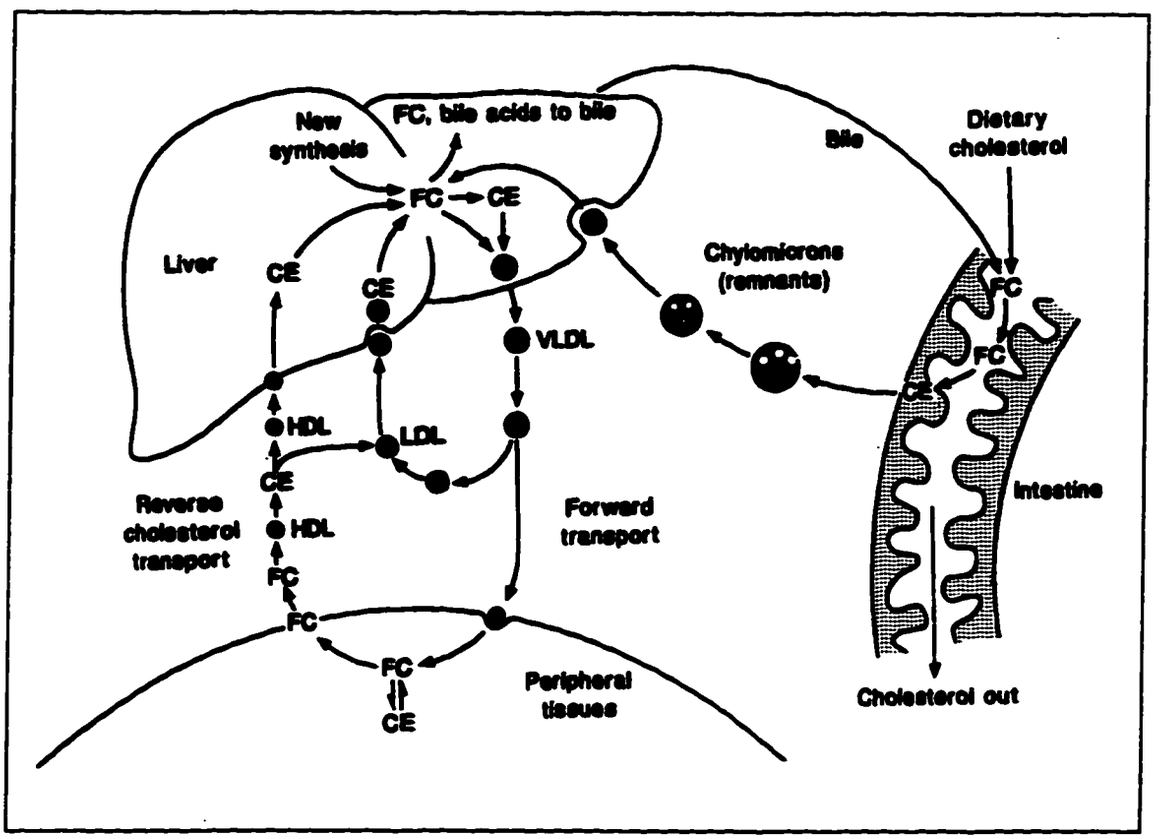



**Fig. 1** ***Major Cholesterol Transport Pathways between the Liver and the Peripheral Tissues***

Exogenous (dietary) cholesterol is absorbed from the intestine as CE and transported by chylomicrons to the liver for catabolism. The liver secretes cholesterol either as bile acids through bile into the intestine or as VLDL into plasma, which are rapidly hydrolyzed into LDL. Thus the remnants of VLDL and LDL deliver endogenous (hepatic) cholesterol to the peri pheral tissues. LDL itself are taken up by the liver. Cholesterol accumulated in peripheral tissues is eventually released to HDL which carries it to the liver for catabolism. Arrows indicate the direction of net transport. (Cited from Fielding and Fielding, 1995).

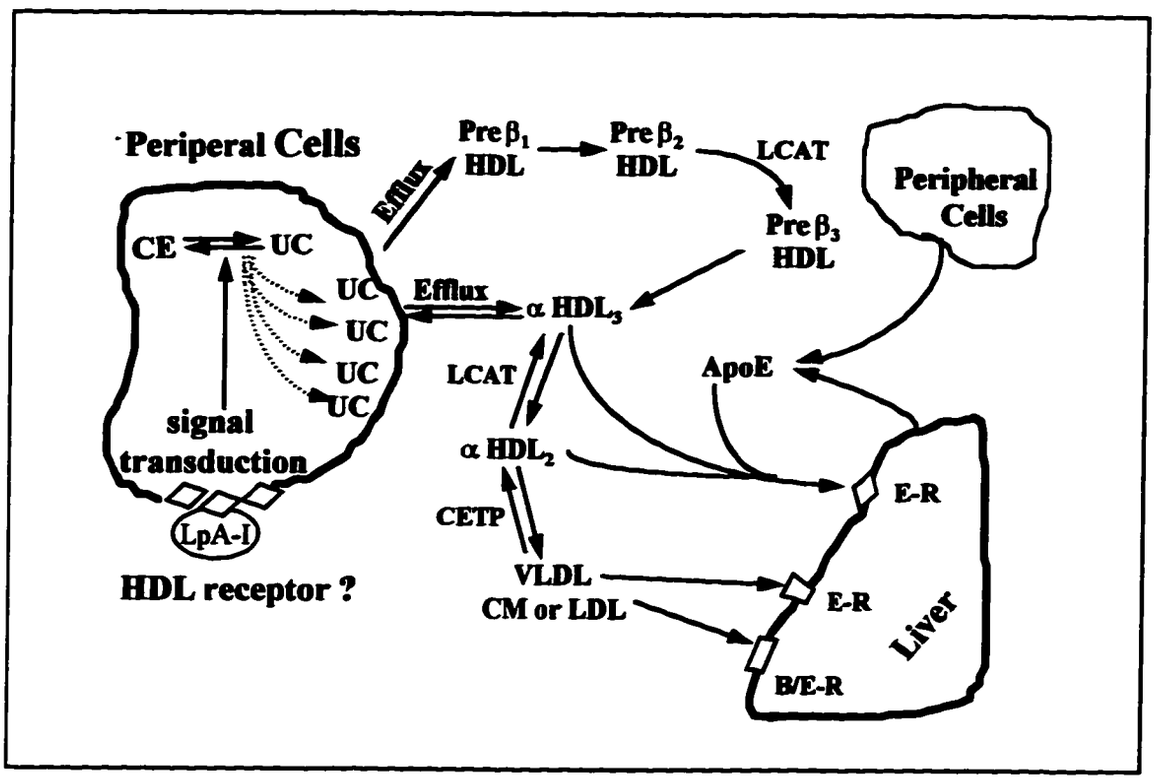



**Fig. 2.** ***The Pathway of Reverse Cholesterol Transport***

This pathway which carries cholesterol from the peripheral tissues to the liver for catabolism, consists of three steps: i) the efflux of cellular cholesterol to initial extracellular acceptors such as pre $\beta_1$–HDL, $\gamma$–LpE, LpA-IV and $\alpha$–HDL; ii) the esterification of free cholesterol into cholesteryl ester by LCAT; iii) The uptake of cholesteryl ester from HDL by the liver via apoE receptor, or from LDL and VLDL via apoE and/or apoB/E receptors. The recipient(s) of subsequent transfer of cell-derived cholesterol from $\gamma$–LpE or LpA-IV are currently unknown.

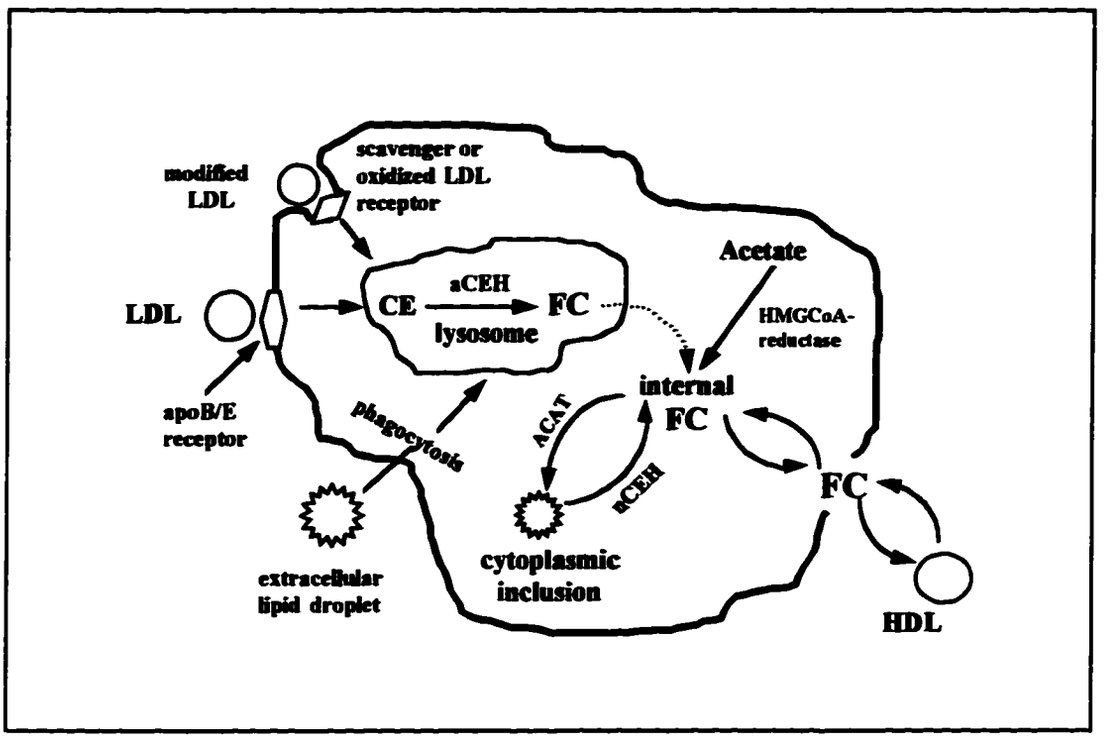



**Fig. 3.** ***Cholesterol Homeostasis in Mammalian Peripheral Cells***

Cells acquire cholesterol by endocytic uptake of lipoproteins and extracellular lipid droplets (Influx), and by de novo biosynthesis (conversion of acetate to cholesterol). The rate of biosynthesis and lysosomal degradation of exogenous cholesteryl ester contributes to the balance of internal pools of free cholesterol. There is continual exchange of cholesterol between the internal pools and cytoplasmatic cholesteryl ester droplets via a cycle of reactions catalyzed by ACAT and neutral cholesteryl ester hydrolase (nCEH). Cholesterol leaves cells by efflux from plasma membrane to HDL particles in interstitial fluid/lymph and plasma. The movement of cholesterol from internal pools probably requires preliminary transport to the plasma membrane.

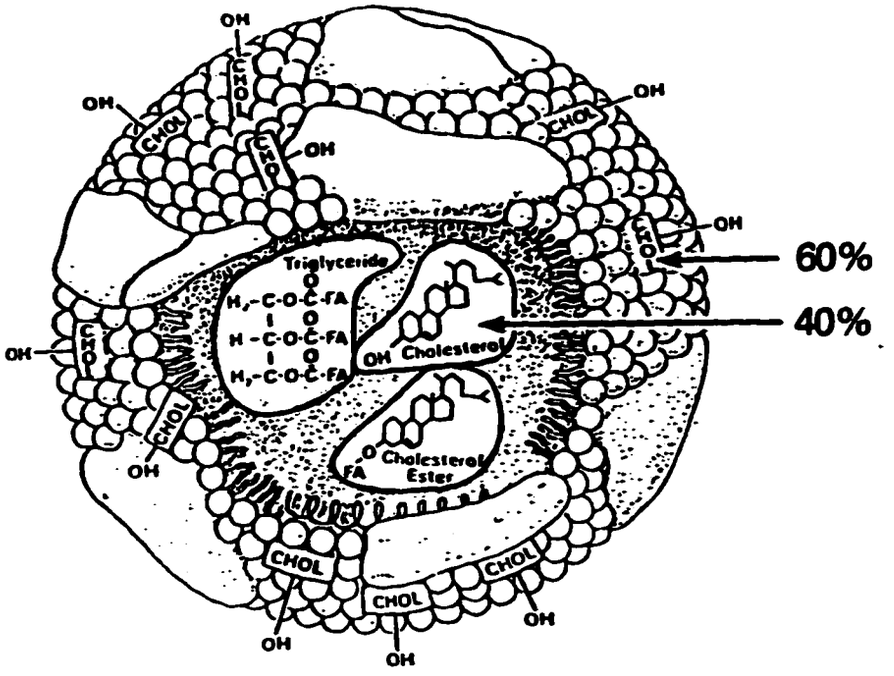



**Fig. 4.** ***Structure of Spherical High Density Lipoprotein***

The surface layer of apolipoprotein and amphiphilic lipid molecules encapsulates a nonpolar core of cholesterol ester, triglyceride, and cholesterol molecules. The polar groups of the phospholipid molecules are all exposed to the aqueous phase; 40% of the unesterified cholesterol molecules are solubilized in the apolar core while the remaining 60% are associated with phospholipid molecules on the surface. Cholesterol molecules exchange between the surface and core locations. (Cited from Lund-Katz et al., 1984)

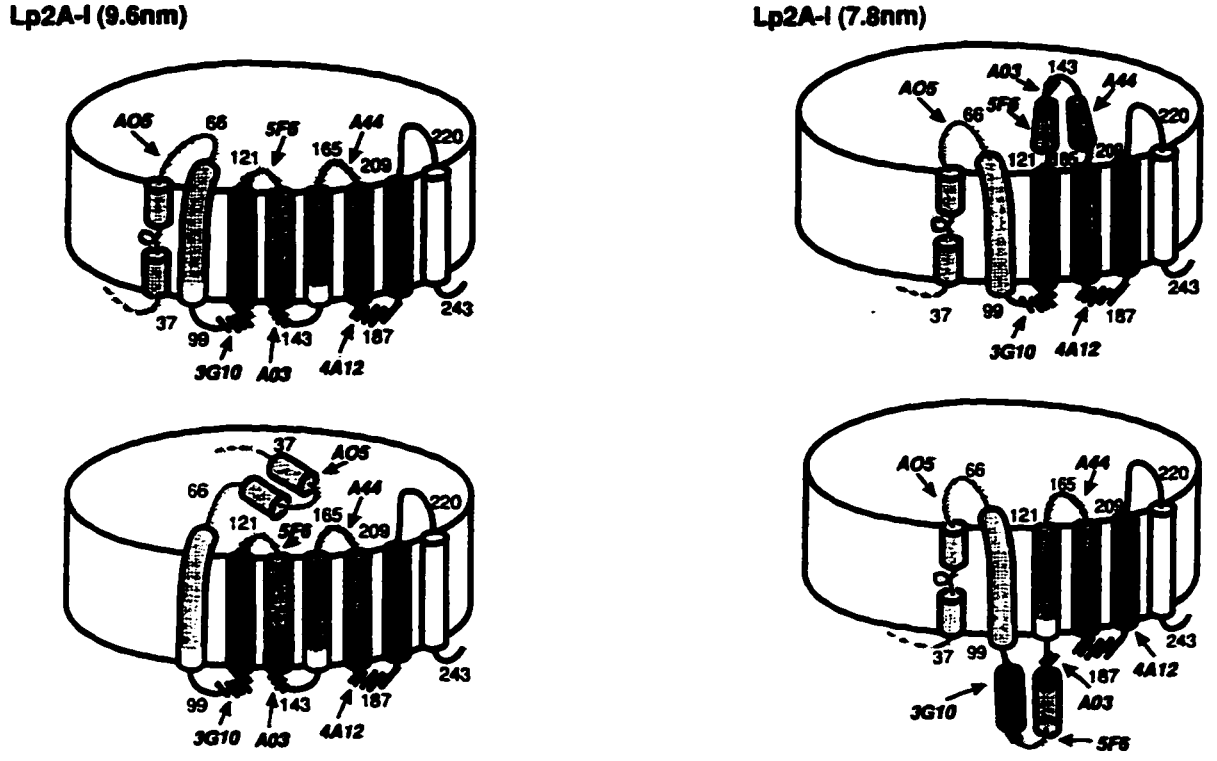



**Fig. 5.** ***Structure of Reconstituted Discoidal LpA-I***

Phospholipids form a bi-layer disc with their hydrophobic acyl chains toward the lipid core and their hydrophilic moiety toward the aqueous phase. Apolipoprotein A-I associates with the edge of the phospholipid disc by interaction with its amphipathic α-helical segments. In larger reconstituted discoidal LpA-I (9.6 nm), apoA-I folds into 7-8 helixes with its N-terminal domain capable of binding either to the edge or to the top of the disc, while in smaller LpA-I (7.8 nm), the sequences between amino acid residues 121-165 or 99-143 fold into hinged domains. (Cited from Calabresi et al., 1993.)

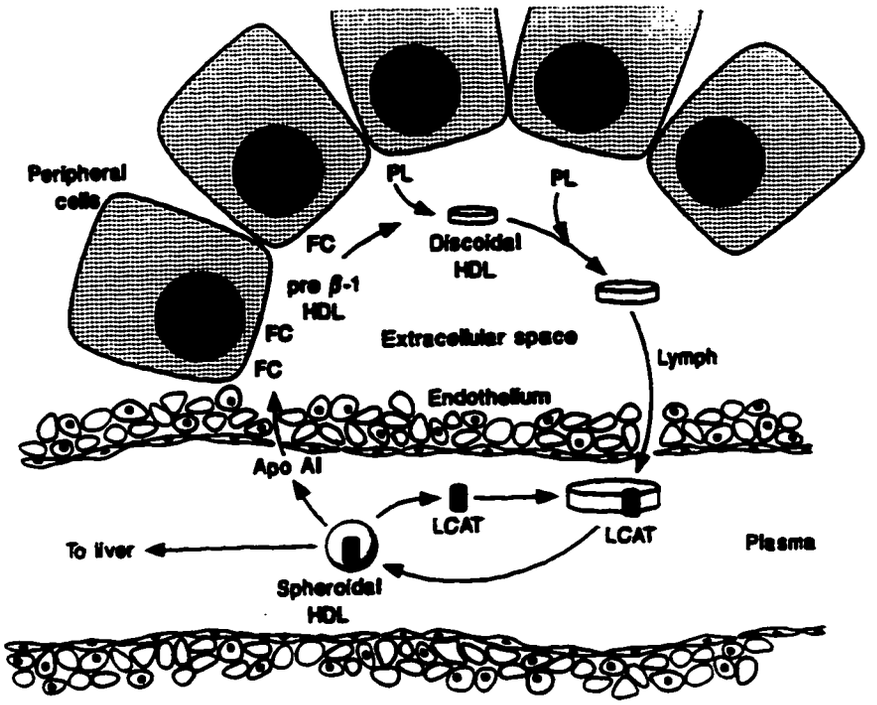



**Fig. 6.** ***The Assembly and Conversion of Pre β-HDL in Extracellular Fluid***

Lipid-free apoA-I passes through endothelial cells and attracts phospholipids and free cholesterol from the peripheral tissue cells to form lipid-poor pre $\beta_1$–HDL complexes which do not have a defined shape. The pre $\beta_1$–HDL can be converted into a discoidal HDL upon progressive incorporation of lipids, then to be released to the circulation. By action of LCAT in plasma, the discoidal HDL will be transformed into spherical particles, which are able to release again their apoA-I component by the actions of several plasma factors. (Cited from Fielding and Fielding, 1995).

# OBJECTIVES

The review of literature clearly illustrates the central role played by HDL in cholesterol transport and in the protection which it affords against atherosclerosis. However after near three decades of active investigations, the high heterogeneity of this lipoprotein class and the complex function of its different subspecies in transporting cholesterol still requires much additional work.

Our initial purpose in this study is to establish a procedure to assay cellular cholesterol efflux during short incubations under conditions where the cholesterol-acceptor lipoproteins remain intact and relatively unmodified by their interaction with the cells, thus allowing assessment of their intrinsic properties. It is also our contention that the measurement of the initial rates of cholesterol efflux is more important physiologically than the long term incubations favored by others. In the course of this work, the common use of albumin as a carrier protein, and the reports by others of its potential to bind cholesterol lead us to study in detail the contribution of albumin to the transport of cholesterol between cells and lipoproteins.

Our main purpose is to understand what are the characteristics of an HDL particle that determine its efficiency as an acceptor of cell-derived cholesterol. Our approach has been to use in vitro reconstituted lipoprotein particles containing

apoA-I, the main HDL apolipoprotein, and the main lipid constituents. Several series of reconstituted HDL-like particles with distinct composition and physical characteristics are therefore prepared by different assembly techniques. The functions of these particles to promote cellular cholesterol efflux will be determined, and the correlations between their functions in efflux and the their physico-chemical characteristics will be analyzed.

# CHAPTER 1

## Materials and Methods

### Materials

Bovine brain sphingomyelin, 1-palmitoyl 2-oleoyl phosphatidylcholine and bovine liver phosphatidylinositol were purchased from Avanti Polar Lipids Inc. (Birmingham, AL). Free cholesterol (+99% grade), triolein, cholesteryl linoleate, gelatin, ovalbumin and essentially fatty-acid free bovine or human serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). Polyvinylpyrrolidone (PVP), boric acid were from BDH Inc. (Toronto, Canada), and guanidine-HCl was from Bethesda Research Laboratories (Bethesda, MD). L-α[myo-inositol-2-$^{3}$H(N)]-phosphatidylinositol, choline-methyl-$^{14}$C-sphingomyelin and 1-2n-$^{3}$H-cholesterol were from Du-Pont Canada Inc. (Mississauga, Canada) with specific activities of 11 Ci/mmole, 50 mCi/mmole and 52 Ci/mmole respectively. Dulbecco's modified Eagle's medium, fetal bovine serum, L-glutamine and penicillin-streptomycin used for cell culture were purchased from GIBCO (Grand Island, NY). All other reagents were analytical grade.

## General Methods:

### 1. Isolation of HDL and Purification of Apolipoprotein A-I

*1.1. Isolation of HDL* --- Fresh plasma was obtained from normolipidemic blood donors, and HDL was isolated by a standard sequential ultracentrifugal technique as described earlier (Schumaker and Puppione, 1986). The plasma was first adjusted with anhydrous potassium bromide (KBr) to a density of 1.063 g/ml and centrifuged at 40,000 rpm for 20 h (55.2 Ti rotor, Backman L8-M ultracentrifuge). The top fraction was removed as LDL and VLDL, and the d > 1.063 g/ml fraction was re-adjusted to a density of 1.21 g/ml and centrifuged again at 40,000 rpm for 40 h to remove other proteins. HDL fraction was then pooled and dialysed at 4°C against 4 liters of fresh saline (0.9% NaCl and 2 mM EDTA), which was changed every 2-3 h for the first two dialyses and overnight for the last one. The HDL was concentrated to a protein concentration of 20-40 mg/ml using centriprep-30 (Amicon Inc. Beverly, MA) before delipidation.

*1.2. Delipidation of HDL and purification of apoA-I* --- The delipidation of HDL was conducted according to the method of Scanu and Edelstein (1971). Briefly, 4-5 ml of HDL at the above concentration was slowly added to 80 ml per-overnight-chilled ether in 250 ml erlenmeyers with gentle shaking, then 120 ml cold ethanol was added slowly against the wall of the flasks so that it did not mix with the ether. When the protein in HDL formed a finely divided precipitate in the

homogenous mixture, the flask was sealed and incubated at -20$^0$C for 5 - 6 h. The solvent was carefully removed by decanting the supernant while leaving most of the precipitate at the bottom of the flask. Then 150 ml ether was added to the flask to mix with the precipitate, and filtered with Whatman No.1 filter. When most of the solvent mix has passed through the filter, the ether/precipitate mix was poured onto the filter, to which another 250 ml ether was added and allowed to filter again until dry. The dried precipitate was scraped into 50 ml Falcon tubes to which 50 ml of ether was added, and tubes were sealed and left at 4$^0$C for overnight. The tubes were then spun at 5,000 rpm for 15 min (Sorvall RT6000D, Du Pont). The supernant was discarded and the precipitate was dried under a gentle stream of nitrogen.

The HDL apolipoproteins were weighed and resuspended in 5 M guanidine-HCl with 30 mM Tris-HCl (pH 8.0) and 0.1% β-mercaptoethanol. The protein concentration in the suspension was analysed by the Lowry method (Lowry et al., 1951), and approximately 60 mg of protein was loaded onto a pre-equilibrated Sephacryl S-200 column (Phamacia) under non-reducing condition using an elution buffer of 5 M urea/1.51 M NaCl/1 mM EDTA/ 10 mM Tris/0.2% $NaN_3$ (pH 8.6). The fractions corresponding to apoA-I were collected and lyophilized, and the purified apoA-I was stored at -70$^0$C. Prior to use, purified apoA-I was resolubilized in 6 M guanidine-HCl and then dialyzed extensively against the

reconstitution buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 1 mM $NaN_3$ as described by Brewer et al (1986) to remove the guanidine.

## 2. Preparation of Reconstituted Discoidal LpA-I with Varying Number of ApoA-I

Reconstituted discoidal HDL lipoproteins containing respectively 2, 3, or 4 molecules of apoA-I per particle were prepared by sodium cholate dialysis method described by Jonas and colleagues (1986). 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC) was used as the source of phospholipid at an initial molar ratio of 120/1/120 (POPC/apoA-I/sodium cholate). Briefly, an appropriate amount of 20 mM POPC in chloroform was added to a 12 x 75 mm glass tube and dried under nitrogen, then sodium cholate at a concentration of 50 mg/ml was added and vortexed vigorously until all the dried phospholipid at the bottom of the tube was resuspended. The tube was sealed and incubated at 4°C until the containing mixture became clear, then apoA-I was added to the solution and incubated at 4°C for overnight. The mixture containing three different types of particles (particles containing respectively 2, 3 and 4 molecules of apoA-I per particle) was separated by gel filtration on a two-serial Agarose columns (95 x 2.5 cm) (BioGel 5M, Bio-Rad Laboratories) running by gravity at a flow rate of 0.20-0.22 ml/min. Peak fractions corresponding to these LpA-I particles were collected,

and concentrated to a final concentration of about 1 mg protein/ml using centriprep-30 (Amicon Inc. Beverly, MA). All the procedures were done in reconstitution buffer as mentioned before. The particles were stored at $4^0$C no more than 2 weeks before being used for efflux study.

### 3. Preparation of Reconstituted Discoidal Lp2A-I with Varying Lipid Composition

All the reconstituted discoidal LpA-I containing 2 molecules of apoA-I per particle but varying POPC and/or other lipids were prepared by sodium cholate dispersion/Bio-Beads removal technique previously described by Sparks et al (1992a, b). As mentioned above, desired amount of 20 mM POPC in chloroform was added and dried under nitrogen into a thin layer at the bottom of a 12 x 75 mm glass tube. Reconstitution buffer containing 10 mM Tris-HCl and 1 mM EDTA (pH 8.0) was added to the tube to give a POPC concentration of 20 mM, and vortexed vigorously for 3 min or more to resuspend all the dried lipids. Sodium cholate at a concentration of 30 mg/ml in reconstitution buffer was added to the tubes at a molar ratio of POPC/cholate of 0.74 and vortexed again for 3 min. The tube was sealed and incubated in a 37°C water bath with vortexing every 15 min for total 2 h until the solution became completely clear. The desired amount of apoA-I was added, and the mixture was diluted to 1 mg protein/ml with

reconstitution buffer and then incubated again at 37°C for 1 h. Sodium cholate was removed from the reconstituted discoidal Lp2A-I mixture by incubation with hydrated Bio-Beads (Bio-Beads SM-2, absorbent 20-50 mesh, Bio-Rad Laboratories) at a ratio of 1 g Bio-Beads/2 mg cholate for 2.5 h with mixing at 4°C, then Bio-Beads were separated by filtration through a 0.22 μm syringe tip filter (Millex-GV, Millipore). After cholate removal, Lp2A-I particles were reisolated by chromatography on a 50 x 2.5 cm Superose-6 gel (Pharmarcia) column running at a flow rate of 0.25 ml/min. The fraction collection and the measurement of particle concentration were performed as mentioned above, and the particles were stored a maximum of one week before being used for efflux study. For the preparation of discoidal Lp2A-I with varying unesterified cholesterol (UC), phosphatidylinositol (PI) or sphingomyelin (SM) contents, appropriate amounts of the respective lipids were mixed and dispersed together with POPC at the beginning of the preparation and the subsequent processing was the same as described above. Protein and phospholipid recoveries were generally greater than 85% in these preparations. In a control experiment, the effectiveness of cholate removal with Bio-Beads was assessed by labeling the sodium cholate with a $^{14}$C-labelled cholate. After the $^{14}$C-cholate containing reconstitution mixture was incubated with the indicated amount of Bio-Beads for 2.5 h at $4^0$C,

radioactivity determination showed that the absorption of the labelled cholate by Bio-Beads was greater than 99%.

**4. Preparation of Reconstituted Sonicated POPC/ApoA-I Complexes**

Reconstituted sonicated POPC/apoA-I complexes were prepared by co-sonication of POPC, apoA-I and other lipids as described by Sparks et al (1995a). Briefly, POPC in chloroform was dried under nitrogen into a 12 x 75 mm glass tube, and 900 μl of reconstituted buffer was added, and vortexed vigorously for 3 min to resuspend the POPC. The lipid mixture was initially sonicated for 1 min at 100% duty cycle (DC) using a Bronson 450 sonicator with one-eight-inch tapered microtip probe and an output control setting at 3 (manufacturer rated output of 40 watts) to disperse the lipids in the aqueous phase. The suspension was then incubated in sealed tubes in a water bath at 37°C for 30 min, and sonicated again at 95% DC for 5 min. Appropriate amount of apoA-I at a concentration of 1.4 mg/ml was added to the lipid suspension, and sonicated again for 4 x 1 min at 90% DC punctuated by 1 min cooling periods. All the sonications were performed in the 12 x 7.5 mm glass tube in a 15°C water bath and under nitrogen to prevent the oxidation of lipids. The resulting mixtures were passed through a 2.2 μm syringe tip filter (Millex-GV, Millipore), and reisolated by size exclusion chromatography on a 50 x 2.5 cm Superose-6 gel (Pharmacia) column running at a flow rate of 0.25

ml/min. As above, for the preparation of sonicated LpA-I particles containing UC, PI, SM, cholesteryl ester (CE) and/or triolein (TG), appropriate amounts of these components were mixed together with POPC at the beginning of the preparation, and processed as described for the particles containing POPC/apoA-I only. All the particles were used for efflux study immediately after preparation since the particles were less stable compared to discoidal LpA-I particles.

## 5. Characterization of Reconstituted LpA-I Particles

*5.1. Homogeneity and size* --- Non-denaturing gradient polyacrymide gel electrophoresis was used to assess the homogeneity, and to estimate the Stoke's diameters of reconstituted LpA-I particles. The particles (1-1.5 μg protein) were applied to 8-25% precast non-denaturing gradient polyacrymide gels (NDGGE, Pharmacia Biotech Inc. Phast Gel), and electrophoresed for 350 Volt.h at $15^0$C, (300 Volts, 10.0 mA). The gels were then stained with Coomassie blue, and the electrophoretic migrations of the particels were measured, from which their Stoke's diameters were calculated from a quadratic equation derived from a polynomial regression of the Stoke's diameters versus the migration distances of five standard proteins (HMW, Phamiacia): thyroglobulin (17.0 nm), apoferritin (12.2 nm), catalase (10.4 nm), lactate dehydrogenase (8.16 nm) and bovine serum albumin (7.1 nm) (Nichols et al., 1986).

*5.2. Electrophoretic mobility and surface potential* --- The electrophoretic mobility of reconstituted LpA-I were determined by electrophoresis on 0.6% precast agarose gels (Beckman, Paragon lipo kit). Samples with 4-6 μg protein were applied per well and allowed to penetrate into the gel for 5 min. The gels were then electrophoresed in an electric field of 100 volts for 30 min at room temperature. After electrophoresis, the gels were fixed for 30 min in a solution containing 30% methanol and 20% acetic acid, dried at 60 $^{0}$C for 1.5 h, and then stained with Coomassie blue. The electrophoretic mobility was calculated by dividing the electrophoretic velocity (migration distance/time) by the electrophoretic potential (voltage applied/gel distance = 18.2 volts/cm) as previously described (Sparks et al., 1992c).

The surface potentials of the LpA-I particles were estimated from their electrophoretic mobility using the most general form of Henry's equation: $S = U6\pi n/D = U \times 19.35$ (Abramson et al., 1942). The equation gives the value of S in mV for a solvent dielectric constant (D) of 78.36 when electrophoretic mobility (U) is expressed in units of $\mu m.s^{-1}.cm.V^{-1}$ and after converting electrostatic volts to ordinary volts.

*5.3. Alpha-helical content* --- The secondary structure of apoA-I in reconstituted LpA-I was monitored by circular dichroism (CD) spectroscopy using

a Jasco J41A spectropolarimeter to measure the percentage of α-helix content in the LpA-I (Sparks et al., 1992c). Reconstituted HDL complexes were diluted with 5 mM phosphate buffer (pH 7.4) to a protein concentration of 66.67 μg/ml, and incubated at 4°C for 72 h before α-helix content was measured at 222 nm.

*5.4. Determination of number of apoA-I molecules per LpA-I particle* --- The number of apoA-I molecules in each reconstituted LpA-I particle was estimated by cross-linking the protein content of the particles with dimethylsuberimidate (DMS) (Swaney, 1986). Briefly, reconstituted LpA-I (25 μg protein) was diluted and dialysed against 0.15 M NaCl to give a final protein concentration of 0.25 mg/ml. The samples were then combined in a ratio of 10:1 (vol/vol) with 20 mg/ml DMS (10-12Å span) freshly disolved in 1 M triethanolamine HCl (pH 9.7). The mixture was incubated at room temperature for 90 min, then lyophilized (Speed Vac SC110, Savants). The sample was resuspended with 25 μl of sample buffer containing 0.5 M Tris-HCl, pH 6.8, 20% glycerol, 10% SDS, 5% mercaptoethanol, and 0.5% (w/v in water) of bromophenol blue (pH 6.8), and heated at 100 °C for 5 min before application to 8 - 25% gradient SDS/PAGE to determine the extent of oligomer formation. Free apoA-I was used as a control.

*5.5. Protein and lipid composition* --- The protein concentration was determined by the Lowry method (Lowry et al., 1951), and the contents of UC, total cholesterol (TC), phospholipids (PL) and TG of LpA-I were measured with commercial enzymatic test kits (Boehringer Mannheim GmBH, Mannheim, Germany). The PI and SM contents of LpA-I were determined in representative preparations by inclusion of either $^{3}$H-PI or $^{14}$C-SM as tracer. The purities of these tracers have been confirmed by 5 x 20 cm K6 silica gel thin layer chromatography (Whatman, New Jersey) using a mixture of chloroform/methanol/ acidic acid/$dH_2O$ (at a volume ratio of 50/30/8/4) as elution solution.

## 6. Cholesterol Efflux from Human Skin Fibroblasts:

*6.1. Cell culture* --- Normal human skin fibroblasts (strain GM0038) were purchased from Clonetics Inc. (Camden, NJ) at their 9th passage. The cells were maintained at 5% $CO_2$ , 95% air and at 37°C in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin and 0.4 mM L-glutamine. Cells used in this study were between the 16th and 25th passages.

*6.2. Labelling of fibroblasts with $^{3}$H-cholesterol* --- Cells were seeded in Falcon 12 well cell culture plates at a density of 5.5 x $10^4$ cells/well, and grown in regular culture condition for 2 days to reach 70% confluence. The cell monolayers

were then washed twice with phosphate buffered saline (PBS), pH 7.4 containing 0.2% BSA and incubated for 5 min with each wash. Following two other washes with PBS alone, the cells were incubated for 48 h with a labelling medium containing 1, 2n-$^{3}$H-cholesterol (20 μCi/well or 50 μCi/well), 5% FBS and other regular supplements for cell culture.

*6.3. Efflux of cellular cholesterol from fibroblasts* --- The human skin fibroblasts prelabelled with $^{3}$H-cholesterol cells were washed twice with DMEM containing 0.2% essentially fatty acid-free BSA and twice with DMEM alone. The washed cells were then incubated with 650 μl of DMEM containing indicated LpA-I particles. At indicated time intervals, aliquots of 50 μl medium were taken and mixed with 100 μl of PBS containing 0.2% essentially fatty acid-free BSA. The mixtures were centrifuged at 10,000 rpm for 5 min to precipitate any detached cells, and 100 μl of the supernatant was used for radioactivity determination. At the end of the incubation, the cell monolayers were washed twice with cold (4$^{0}$C) PBS containing 0.2% essentially fatty acid-free BSA and twice with PBS alone at room temperature, then were lysed and solubilized by overnight incubation with 0.5 ml of 0.1 N NaOH. The radioactivities of cell lysate and media were quantified by liquid scintillation spectrometry (Wallac 1409). The protein concentrations of the cell lysate were determined by the Lowry method. The specific activity of cell

cholesterol was 1.0-1.3 x $10^6$ cpm/μg cholesterol, and more than 90% of the radioactivity was in the UC fraction as previously reported (Castro and Fielding, 1988). Efflux was expressed as cpm of $^3$H-cholesterol in medium per microgram cellular protein. Incubation in the absence of cholesterol acceptor was used as control for efflux.

## 7. Incorporation of Cholesterol into Essentially Fatty Acid-free Human Serum Albumin by Sonication

The incorporation of cholesterol into essentially fatty acid-free human serum albumin (HSA) was performed basically as described for the labelling of reconstituted HDL-like particles (Jonas et al., 1989). $^3$H-cholesterol (specific activity is 178.7 μCi/μmole UC) at a molar ratio of 2.5/1 to albumin was dried under nitrogen. Cold acetone at 100 μl was added to dissolve the cholesterol, then the acetone-cholesterol mixture was added drop-wisely during vortexing into a HSA solution (10 mg/ml in a recombination buffer). After evaporation of the acetone under nitrogen, the mixture was sonicated for 1 min (energy output of 40 watt, 100% DC) and incubated at 37°C in a water bath for 1.5 h, then sonicated 3 × 1 min again at 90% DC. The non-incorporated cholesterol was removed first by passing through a 0.45 μm syringe tip filter (Millex-HA, Millipore), and then by size exclusion chromatography on a 50 × 2.5 cm Superose-12 (Phamarcia) column

running at a flow rate of 0.25 ml/min. Radioactivities of the fractions corresponding to the protein peak of HSA determined by absorbance at 280 nm were also determined by liquid scintillation counting (Wallac 1409) in order to evaluate the co-distribution of these two molecules. The fractions were then pooled together and concentrated with a Centriprep-30 concentrator (Amicon Inc. Beverly, MA) into a preparation that has a specific activity of $2.01 \times 10^8$ cpm/μmole cholesterol or $4.81 \times 10^6$ cpm/μmole HSA.

## 8. Preparation of Reconstituted Discoidal LpA-I Particles Containing $^3$H-Cholesterol

Reconstituted discoidal HDL-like particles containing $^3$H-cholesterol were prepared by the sodium cholate dispersion method described above. The preparation was started with an initial molar ratio of POPC/apoA-I/UC at 80:1:4. Appropriate amount of 1, 2n-$^3$H-cholesterol at a specific activity of 297.7 μCi/μmole was mixed with other lipid components at the beginning of the preparation. The preparation was then processed as described before. The specific activity of the reisolated LpA-I particles was $3.34 \times 10^8$ cpm/μmole cholesterol or $9.64 \times 10^8$ cpm/μmole apoA-I.

## 9. Transfer of Cholesterol from HSA-($^3$H)-Cholesterol Complexes or LpA-I-($^3$H)-Cholesterol to Fibroblasts

Cells were seeded into Falcon 12 well cell culture plates at a density of 5.5 × $10^4$ cells/well four days before the transfer experiment. After 48 h, the cells were washed as previously mentioned for efflux experiments and changed to a medium containing 5% FBS and other supplements but no 1,2n-$^3$H-cholesterol for another 48 h. The cells were then washed twice with DMEM containing 0.2% essentially fatty acid-free BSA and twice with DMEM alone. Transfer was started by adding to the cell monolayers the medium containing either LpA-I labelled with $^3$H-cholesterol at a protein concentration of 45 μg/ml or HSA-($^3$H)-cholesterol complexes at a protein concentration of 2 mg/ml. The incubation was carried out at 37°C with gentle shaking as described for the cholesterol efflux study. Media were removed from individual wells after 2, 5, 15, 30, 60, and 90 min of incubation. The cells were washed twice with PBS containing 0.2% essentially fatty acid-free BSA and twice with PBS alone, then were lysed in 0.5 ml of 0.1 N NaOH. Aliquots of the cell lysate were used for radioactivity counting and for protein assay. Cholesterol transfer was calculated as the percentage of original medium radioactivity that has been transferred into cells, and as cholesterol mass delivered to the cells.

In another experiment, the LpA-I-($^3$H)-cholesterol, or HSA-($^3$H)-cholesterol complexes used for the influx study were obtained from the incubation of HSA or LpA-I with ($^3$H)-cholesterol labelled fibroblasts. The preparation procedure was as follows: Cells were seeded, washed and labelled as for cholesterol efflux, and the cells were then incubated at 37°C with DMEM containing either 2 mg/ml of HSA or 45 μg protein/ml of reconstituted discoidal LpA-I. The media were collected at 3 h of incubation and centrifuged at 10,000 rpm for 5 min to remove detached cells. The cell-conditioned media were then added to new plates where unlabelled fibroblasts had undergone the same seeding, washing and culture procedures. After either 15 min or 90 min of incubation, the media were taken out, the cells in the corresponding wells were washed and lysed as mentioned above and aliquots of media were used for scintillation counting and protein assay.

## 10. Transfer of Cholesterol from HSA-($^3$H)-Cholesterol Complexes to Fibroblasts in the Presence of either LDL or Reconstituted LpA-I

The transfer of cholesterol from HSA-($^3$H)-cholesterol complexes to fibroblasts was studied in the presence of either LDL or reconstituted LpA-I at concentrations up to 80 μg protein/ml. In the study with LDL, two monoclonal antibodies 4G3 and 5E11 were added, each in 3 molar excess relative to LDL-apoB, in order to prevent the binding of LDL to the LDL receptors of fibroblasts.

These monoclonal antibodies have been reported to interact with amino acid residues 2980-3084 and 3441-3569 of apoB respectively, and are able to block the binding of LDL to their cell surface receptors (Milne et al., 1989). The antibodies were pre-incubated with LDL for 1.5 h at $37^0$C, and then added immediately before the influx study. The influx incubations were carried out with a gentle shaking as in the efflux study, and media were taken out at 90 min incubation. Cell washing and radioactivity determinations were the same as described above. In the incubation with reconstituted LpA-I, no antibody was added to the medium since our preliminary study has demonstrated that the binding/uptake of the LpA-I in fibroblasts was negligible during a 90 min incubation.

## 11. Determination of ApoA-I and Fatty Acids in Different Human Serum Albumin Preparations

11.1. *ApoA-I radioimmunoassay* --- The presence of apoA-I in different HSA products was first assayed by the solid-phase radioimmunoassay described by Calabresi et al (1993). Briefly, Immulon II Removawells were coated with 0.2 μg apoHDL in 100 μl coating buffer containing 15 mM $Na_2CO_3$/35 mM $NaHCO_3$/ 0.02% $NaN_3$ (pH 9.6) at $4^0$C for overnight in a moist environment. After washing once with PBS, the wells were saturated with 300 μl of gelatin (0.5% in PBS, pH 7.2) at room temperature for 60 min. Monoclonal antibody 4H1 at 1:6000 and

1:3000 dilution respectively was mixed with either a serially diluted essentially fatty acid-free HSA (from 2 μg to 4 mg) or a serially diluted purified lipid-free apoA-I (from 29 ng to 60 μg) in reaction buffer (0.5% gelatin/0.02 $NaN_3$ in PBS, pH 7.2), and added to the coated and saturated wells, then incubated for 1 h at room temperature. After discarding the incubation solution, the wells were washed 3 times with 0.05% Tween, 0.02% $NaN_3$ in PBS, pH 7.2. Finally, 100 μl of $^{125}I$-labelled rabbit anti-mouse IgG in reaction buffer was added to each of the wells, and incubated again for 1 h at room temperature. The wells were washed extensively and counted on a Packard COBRA II γ counter. The apoA-I contents in HSA products were calculated from the $B/B_0$ of the serially diluted purified lipid-free apoA-I, where B and $B_0$ represent the cpm bound in the presence and absence of competitive antigen.

11.2. *Polyacrylamide gel electrophoresis and immunoblots* --- Each of the different HSA preparations at 50 μg, and purified lipid-free apoA-I at 0.5, 1 and 1.5 μg were electrophoresed on a 4-20% non-denaturing polyacrylamide gel (mini-protean II ready gel, Bio-Rad) at 100 volts for 3.5 h, and stained with Coomassie blue. The proteins from a duplicate gel were transferred to a nitrocellulose membrane at $4^0C$, 350 mA for 2 h, in a solution containing 30% methanol and 43.7 mM boric acid, pH 8. The nitrocellulose was saturated with 3%

polyvinylpyrrolidone (PVP) in Tris-base buffered saline (TBS) (150 mM NaCl, 10 mM Tris-base, pH 7.4, 0.02% $NaN_3$) for 60 min at 37$^0$C, then incubated for 1.5 h at 37$^0$C with gentle shaking with a mouse anti-apoA-I IgG 4H1, a mAb against the N-terminal 2-8 amino acid residues of apoA-I (Marcel et al., 1991) at 1:1000 dilution. The nitrocellulose was then rinsed extensively with TBS, and incubated with $^{125}$I-labelled rabbit anti-mouse IgG ($1 \times 10^6$ cpm/ml) in 3% BSA-TBS for 1 h at 37$^0$C with gentle shaking. After extensive washing with TBS, the membrane was air dried and exposed to XAR-5 Kodak film for overnight at -80$^0$C to estimate the apoA-I contents.

11.3. *Determination of fatty acids in HSA* --- Determination of fatty acids in the different HSA preparations was carried out by first extracting lipids from the HSA products using the method of Bligh and Dyer (1959), then the extracts were applied to gas chromatograph (Hewlett Packard 5890 series II plus) using C17:0 methyl ester as internal standard. The data were expressed as μg of total fatty acids per mg of protein.

# CHAPTER 2

## Serum Albumin is a Significant Intermediate in Cholesterol Transfer between Cells and Lipoproteins

### I. SUMMARY

The function of albumin in the movement of cholesterol in and out of non-cholesterol loaded fibroblasts has been investigated. The results show that cholesterol efflux from cholesterol labelled normal human skin fibroblasts to fatty acid-free human serum albumin (HSA) is bi-phasic with a rapid first phase that plateaus at about 15 min followed by a nearly linear phase up to 90 min, the longest incubation in this study. Saturation of efflux is observed at both 60 and 90 min at a concentration of about 10 mg albumin/ml. The function of albumin in efflux is specific since other molecules, such as ovalbumin or gelatin, have no effect and ovalbumin even slightly reduces the efflux compared to DMEM alone. The ability of HSA to induce cellular cholesterol efflux is low compared to reconstituted discoidal lipoprotein A-I (LpA-I). HSA at 2 mg/ml produces a rate of cholesterol efflux similar to that of LpA-I at 45 μg protein/ml, however these concentrations are within the physiological range for both HSA and apoA-I. The efflux to the medium containing both LpA-I and HSA is greater than that to each

of them alone at both 60 ($p<0.05$) and 90 min ($p< 0.01$) incubation, but does not show complete additivity, indicating a competition between HSA and LpA-I. The HSA-mediated cholesterol movement is bi-directional as demonstrated by the transfer of cholesterol from HSA-($^3$H)-cholesterol complexes to fibroblasts. Moreover, the HSA-mediated transfer is much faster than that from cholesterol-containing LpA-I (0.8 versus 0.2 pmole/μg cell protein at 90 min incubation). The presence of either LDL or LpA-I in the incubation medium significantly inhibits the transfer of cholesterol from HSA-($^3$H)-cholesterol complexes to fibroblasts, thus allowing the bi-directional transfer of cholesterol between HSA and cells to possibly operate as a net efflux. The results of this study suggest that albumin may contribute to the delivery of cholesterol to the liver by lipoproteins, and as such plays a significant role in reverse cholesterol transport.

## II. INTRODUCTION

Previous studies have suggested the possible involvement of albumin in cholesterol metabolism and in the process of atherosclerosis. For example, a positive correlation was reported between the plasma total cholesterol/albumin ratio and the plasma total cholesterol/HDL ratio (Nanji, 1983) or between serum albumin and total serum cholesterol (Gillum and Makuc, 1992, Gillum, 1993). Relatively low serum albumin has been suggested as a predictor of increased incidence and mortality for coronary heart disease (Phillips et al., 1989, Gillum and Makuc, 1992, Gillum, 1993).

Albumin contains six hydrophobic domains, and is able to interact with a wide variety of hydrophobic ligands. The major ligands are fatty acids, which are otherwise insoluble in circulating plasma. Each albumin molecule has been reported to be able to bind up to six molecules of fatty acids (reviewed by Carter and He, 1990, Spector, 1986) as well as long-, medium- and short-chain monoacyl glycerol (Wang et al., 1993). In addition, albumin is also able to bind bile acids (Brock, 1976, Roda et al., 1982), steroid hormones and some hydrophobic drugs, such as digitoxin (Brock, 1976). The albumin receptors on the surface of the liver cells take up fatty acids and other albumin-bound substrates (Weisiger et al., 1981, Forker and Luxon, 1981). There is no doubt that albumin is an important multifunctional transport protein in plasma.

Albumin might also play a role in the transport of lipid and cholesterol in plasma. An early report of Fielding and Moser (1982) showed that removal of albumin from plasma by affinity chromatography reduced by more than 50% the ability of plasma to release cholesterol from normal cultured fibroblasts. The study of Mendel and Kunitake (1988) showed a concentration dependent stimulation of cholesterol efflux from the same cells by bovine serum albumin (BSA). Incubation of cholesterol-enriched Fu5AH rat hepatoma or GM 3468 human skin fibroblasts with BSA could significantly reduce the cholesterol content of these cells (Johnson et al., 1991b). The function of albumin in lipid metabolism has been clearly demonstrated in analbuminemic patients, in nephrotic syndrome patients, as well as in Nagase analbuminemic rats (NAR) (Cohen et al., 1980, Baldo-Enzi et al., 1987).

However, in spite of the above evidence for the participation of albumin in the metabolism of lipid and cholesterol, this protein has not been considered as a specific and significant contributor to cellular cholesterol movement and is in fact frequently used as a saturating carrier protein in such studies. This ignorance of the role of albumin in cholesterol efflux has been reinforced by recent characterization of high affinity acceptors for cellular cholesterol which have been obtained using methods which could not register the participation of a bulk carrier such as albumin to this process (Castro and Fielding, 1988, Huang et al., 1994,

von Eckardstein et al., 1995). We demonstrate here that even in short term incubations, albumin specifically mediates a bi-directional movement of cellular cholesterol with low affinity but high capacity. By virtue of its high concentration in plasma, albumin contributes to a significant proportion of cholesterol efflux. Furthermore we show that albumin, like HDL, promotes a multi-directional transfer of cholesterol between cells and extracellular lipoproteins.

# III. RESULTS

## 1. *Cholesterol efflux from fibroblasts to human serum albumin*

The effect of human serum fraction V, essentially fatty acid-free albumin (HSA) on cholesterol efflux from $^{3}$H-cholesterol-labelled, non-cholesterol-loaded human skin fibroblasts is studied initially. The results indicate that this protein is able to promote a significant efflux of cellular cholesterol from fibroblasts during a 90 min incubation as illustrated in Fig. 2-1A, 2-1B. The efflux follows a bi-phasic pattern, a characteristic of cellular cholesterol efflux from human skin fibroblasts which is also observed with medium containing HDL or reconstituted LpA-I (see Chapter 3 and 4). The initial rapid efflux reaches a peak at about 15 min and is followed by a plateau up to about 30 min. The second phase of efflux is linear for up to 90 min, the longest incubation in this study. When fibroblasts are incubated with HSA for 30, 60 or 90 min respectively, the efflux is concentration dependent within a range of 0.5 to 10 mg protein/ml and appears to saturate at a concentration of about 10 mg/ml at both 60 and 90 min of incubation (Fig. 2-2A, 2-2B). The estimated $EC_{50}$ at 60 and 90 min of incubation are 3.87 and 2.56 mg/ml respectively.

It should be emphasized that the ability of HSA to promote cellular cholesterol efflux is much lower than that observed from HDL or reconstituted

Lp2A-I. As shown in Fig. 2-3, HSA at a concentration of 2 mg/ml induces cholesterol efflux rates which are very close to those induced by 45 μg protein/ml reconstituted Lp2A-I at both 60 and 90 min of incubation. The interactions of HSA and LpA-I with cholesterol releasing from cells are probably competitive since the efflux to a medium containing both HSA (2 mg/ml) and Lp2A-I (45 μg protein/ml) is less than the sum of the efflux rates induced by each of them alone. Nevertheless, the efflux to a medium containing both HSA and Lp2A-I is statistically higher than that to the media with each of them alone at both 60 min ($P<0.05$) and 90 min ($P<0.01$) of incubation.

*2. Specificity of cholesterol efflux from fibroblasts to human serum albumin*

To demonstrate that this process represents a specific interaction of cholesterol with albumin, we have also studied the cholesterol efflux mediated by two other molecules, ovalbumin, a non serum albumin, and gelatin, a matrix molecule. Cholesterol efflux to a medium containing either HSA, ovalbumin, or gelatin at the same concentrations of 0.5, 1 and 2 mg/ml are compared. As shown in Fig. 2-4, neither gelatin nor ovalbumin can replicate the stimulating effect of serum albumin on cholesterol efflux, instead, ovalbumin even shows a slightly inhibitory function in this process.

Since the presence of apoA-I in commercial serum albumin products has been reported previously (Fainaru and Deckelbaum, 1979, Deckelbaum et al., 1980), and since apoA-I alone is able to promote the efflux of cholesterol from cells (Forte et al., 1993, Hara and Yokoyama, 1991, 1992, Bielicki et al., 1991, 1992, Huang et al., 1995), the presence of apoA-I in the albumin products used in this study has been analysed. Radioimmunoassay shows apoA-I to be present in the essentially fatty acid-free HSA preparation at less than 0.006 μg apoA-I/mg HSA. This negligible concentration of apoA-I in the HSA preparation used is also corroborated by immunoblot estimation of the HSA separated by SDS/PAGE gel electrophoresis (Fig. 2-5). Therefore this trace amount of apoA-I cannot contribute to the significant efflux mediated by HSA.

The presence of fatty acids in regular fraction V HSA and in essentially fatty acid-free HSA is also measured and the results indicate a fatty acid content of 12.0 and 6.8 μg fatty acid/mg protein respectively. When these different preparations of human serum albumin are compared, cellular cholesterol efflux to regular fraction V HSA is slightly higher than that to the essentially fatty acid-free HSA, which is in agreement with a previous study (Chau et al., 1978). However, since immunoblot had demonstrates the presence of apoA-I in regular fraction V HSA preparation (Fig. 2-5), the higher cellular cholesterol efflux to regular

fraction V HSA may also reflect the contribution of the contaminating apolipoprotein.

### 3. *Transfer of cholesterol from HSA-($^3$H)-cholesterol complexes or LpA-I-($^3$H)-cholesterol to fibroblasts*

Cholesterol transfer between cells and native HDL or reconstituted LpA-I is known to be bi-directional (Johnson et al., 1986); therefore, one should expect that in addition to promoting cellular cholesterol efflux, albumin may also deliver cholesterol to fibroblasts. To verify this possibility, we prepared HSA-($^3$H)-cholesterol complexes by incorporation of tritium labelled cholesterol into HSA molecules by sonication. The HSA-($^3$H)-cholesterol complexes are purified by size exclusion chromatography to remove uncoupled free cholesterol. The overlap of the elution profiles of the radioactivity of $^3$H-cholesterol and protein absorbance at 280 nm demonstrates the homogeneous distribution of cholesterol and albumin, and documents the association of cholesterol with HSA (Fig. 2-6). When the HSA-($^3$H)-cholesterol complexes are incubated with non-cholesterol-loaded fibroblasts, the cellular $^3$H-cholesterol content increases with time (Fig. 2-7), demonstrating that HSA-($^3$H)-cholesterol complexes are able to deliver their cholesterol contents to cells. It is of interest that the transfer rate of cholesterol from HSA to fibroblasts is faster than that from cholesterol-containing reconstituted discoidal LpA-I. At 90

min incubation, the percentage of cholesterol transfer from HSA-($^3$H)-cholesterol complexes to fibroblasts is 13.8%, in contrast to 0.7% from LpA-I-($^3$H)-cholesterol particles (Fig. 2-7A). These represent mass transfers of 0.8 and 0.2 pmole cholesterol/μg cell protein respectively from HSA and LpA-I as calculated from their specific activities (Fig. 2-7B).

In another experiment, HSA or LpA-I are labelled with cholesterol by incubation for 3 h with $^3$H-cholesterol labelled fibroblasts as described in Chapter 1. The cell conditioned media are centrifuged to remove any possibly detached cells, and then transferred to non-labelled cells and incubated for 15 and 90 min respectively in order to measure the transfer of cholesterol from each donor to the cells. The radioactivities associated with HSA (2 mg/ml) or with discoidal reconstituted LpA-I (45 μg protein/ml) after 3 h pre-incubation with fibroblasts are similar ($1.22 \times 10^6$ and $1.13 \times 10^6$ cpm/ml respectively). As observed with the transfer from sonicated HSA-($^3$H)-cholesterol complexes, cholesterol transfer from cell-conditioned HSA-($^3$H)-cholesterol complexes to fibroblasts was faster than that from LpA-I-($^3$H)-cholesterol particles (1.1 versus 0.6 % at 15 min, and 8.1 versus 4.3% at 90 min) (Fig. 2-8).

### 4. *Transfer of cholesterol from HSA-($^3$H)-cholesterol complex to fibroblasts in the presence of LDL or reconstituted discoidal LpA-I*

The transfer of cholesterol from HSA-($^3$H)-cholesterol complexes to fibroblasts is also studied in the presence of either reconstituted LpA-I or freshly prepared LDL at various concentrations up to 80 μg protein/ml. As expected, cholesterol transfer from HSA-($^3$H)-cholesterol complexes to fibroblasts is very susceptible to the presence of either of these lipoproteins. The inhibitory effects of LDL and Lp2A-I on the transfer of cholesterol from HSA-($^3$H)-cholesterol complexes to fibroblasts are dose-dependent, with the $EC_{50}$ less than 5 μg protein/ml, and the maximal inhibitions at about 40 μg protein/ml for both of these lipoproteins (Fig. 2-9).

# IV. DISCUSSION

The results of this study clearly demonstrate that albumin is able to play a role in the transport of cholesterol between cells and lipoproteins. This is in agreement with the earlier report of Fielding and Moser (1982) which showed that removal of albumin from human plasma decreased by about 50% of its ability to promote cellular cholesterol efflux. This effect is specific to serum albumin and cannot be replicated by either an avian protein such as ovalbumin, or a matrix molecule, like gelatin (Fig. 2-4). There is a low level of fatty acids in the HSA preparations used in this study, and we have observed that regular fraction V HSA preparation which contains relatively more fatty acids promotes slightly more cellular cholesterol efflux than fatty acid-free albumin. It is therefore possible that cholesterol may be indirectly associated with albumin through interactions with some of its hydrophobic ligands. On the other hand, the presence of trace amount of apoA-I in the essentially fatty acid-free HSA preparations used, makes it unlikely that apoA-I could have contributed to the albumin-mediated efflux of cholesterol.

It is not unexpected that plasma albumin should be able to bind to cholesterol, and function as a cholesterol transporter. As discussed above, albumin is a multiligand carrier for a large variety of hydrophobic ligands, such as hormones (Brock, 1976), bile acids (Brock, 1976, Roda et al., 1982), hydrophobic

drugs (Brock, 1976), and the most predominant, fatty acids and monoacyl glycerol (Carter and He, 1990, Wang et al., 1993, Spector, 1986). The binding ability of albumin for cholesterol is low as shown here that: i) the in vitro combination of HSA and cholesterol by sonication yields complexes of HSA-cholesterol with a molar ratio of approximately 20/1; ii) HSA at a concentration of 2 mg/ml shows an ability to release cellular cholesterol equivalent to that of LpA-I at 45 μg protein/ml. While this makes apoA-I in LpA-I 19 times more efficient than albumin, the plasma albumin molarity is about 15 folds that of apoA-I. This is therefore compatible with the proposition that albumin can mediate cholesterol efflux to an extent comparable to that of apoA-I. This is also true of peripheral interstitial fluid, where the ratio of albumin to apoA-I was shown to be greater than that of plasma (Wong et al., 1992). The concentration of albumin used here is about 5 to 10 time less than its physiological level, while the concentration of LpA-I protein used was selected to represent the approximate levels of preβ-migrating HDL (Kunitake et al., 1985), thus reinforcing the notion that albumin can be a physiologically effective intermediate of cholesterol efflux .

This study is the first to demonstrate that albumin can mediate a multi-directional transfer of cholesterol between cells and extracellular lipoproteins. In addition to its function in promoting cellular cholesterol efflux, albumin-bound cholesterol can be transferred into cells faster than that from LpA-I (Fig. 2-6). It is

of interest that the transfer of cholesterol from HSA-($^3$H)-cholesterol complex to cells is very sensitive to the presence of LDL or reconstituted discoidal LpA-I in the incubation medium. With the presence of only 5 μg protein/ml of either LDL or LpA-I, the transfer of cholesterol from HSA-($^3$H)-cholesterol complex to cells is inhibited by about 65%. This leads us to hypothesize that plasma albumin can serve as a transient carrier for cholesterol between cells and other lipoproteins. By itself, albumin may be able to promote the efflux of cholesterol from certain extrahepatic cells to form albumin-cholesterol complexes, from where the cell-derived cholesterol is transferred to lipoproteins which have higher affinity for cholesterol. Thus albumin can mediate an initial pathway for the reverse cholesterol transport which may be an alternative to that mediated by pre β–HDL (Castro and Fielding, 1988), γ–LpE (Huang et al., 1994), or LpA-IV (von Eckardstein et al., 1995).

Albumin deficiency results in altered lipid and cholesterol metabolism, such as in congenital analbuminemia and in the nephrotic syndrome characterized by greatly elevated plasma levels of triacylglycerol and cholesterol (Baldo-Enzi et al., 1987, Cohen et al., 1980). The same phenomenon is also observed in the analbuminemic rat, a mutant Sprague-Dawley rat with a post-transcriptional deficiency of albumin mRNA (Nagase et al., 1979, Takahashi et al., 1983, Catanozzi et al., 1994, Van Tol et al., 1991, Joles et al., 1991). A positive

relationship between plasma albumin and HDL cholesterol has also been reported in nephrotic syndrome (Cohen et al., 1980). Some plasma enzymes involved in lipid and cholesterol metabolism have altered activities in analbuminemia. For example, lecithin:cholesterol acyltransferase (LCAT) activity is increased in both analbuminemic patients and NAR (Baldo-Enzi et al., 1987, Van Tol et al., 1991), and hepatic triacylglyceride lipase is slightly decreased (Baldo-Enzi et al., 1987). However, up to now, the mechanism involved in the analbuminemia related disorder of lipid and cholesterol metabolism is still unclear.

ApoA-I containing HDL has been reported to play a central role in cholesterol metabolism through its functions as an acceptor for cell-derived cholesterol and the subsequent esterification of the cell-derived cholesterol. However, although reduced plasma concentration of this apolipoprotein and especially low level of LpA-I have been reported in patients with coronary disease (Kukita et al., 1984, Puchois et al., 1987), deficiency or severe reduction of apoA-I does not increase the risk of coronary disease in mice (Li et al., 1993). Similarly, analbuminemic individuals or analbuminemic rats do not show increased risk for cardiovascular disease (Baldo-Enzi et al., 1987, Joles et al., 1991). This may be related to the compensatory synthesis of other apolipoproteins induced by deficiency of plasma albumin (Joles et al., 1991, Baldo-Enzi et al., 1987); however, the apolipoprotein profiles of analbuminemic subjects indicate that not

all the apolipoproteins increase simultaneously (Joles et al., 1991, Baldo-Enzi et al., 1987, Van Tol, 1991). The specific increase in $HDL_3$ and apoA-I would be expected to compensate for the reduced cellular cholesterol efflux due to lack of albumin since LpA-I is well known to be a good acceptor for cell-derived cholesterol (Mahlberg et al., 1990, 1991, DeLamatre et al., 1986, Stein et al., 1986).

# V. CONCLUSION

It has been shown in this study that albumin is a significant intermediate for cellular cholesterol transport and can contribute to the transfer of cholesterol between cells and lipoproteins, which ensures the eventual return of cell-derived cholesterol to the liver. These properties make albumin a significant protein in reverse cholesterol transport.

**Fig. 2-1.** ***Time Course of Cellular Cholesterol Efflux to HSA***

Human skin fibroblasts were labelled with ($^3$H)-cholesterol (20 μCi/well) in medium containing 5% FBS for 48 h. For the efflux study, the cells were washed twice with DMEM containing 2 mg/ml essentially fatty acid-free BSA, and twice with DMEM alone, and then were incubated with DMEM containing HSA at a concentration of 2 or 5 mg/ml respectively. DMEM without HSA was used as control. Aliquots of medium were taken at 2, 5, 15, 30, 60 and 90 min respectively for determination of radioactivity. Panel A shows the efflux expressed as medium radioactivity per μg cell protein. Panel B shows the efflux expressed as percentage of ($^3$H)-cholesterol in the medium relative to total cellular radioactivity. Each data point presented is the mean of 4 replicate samples and the error bar is the standard deviation .

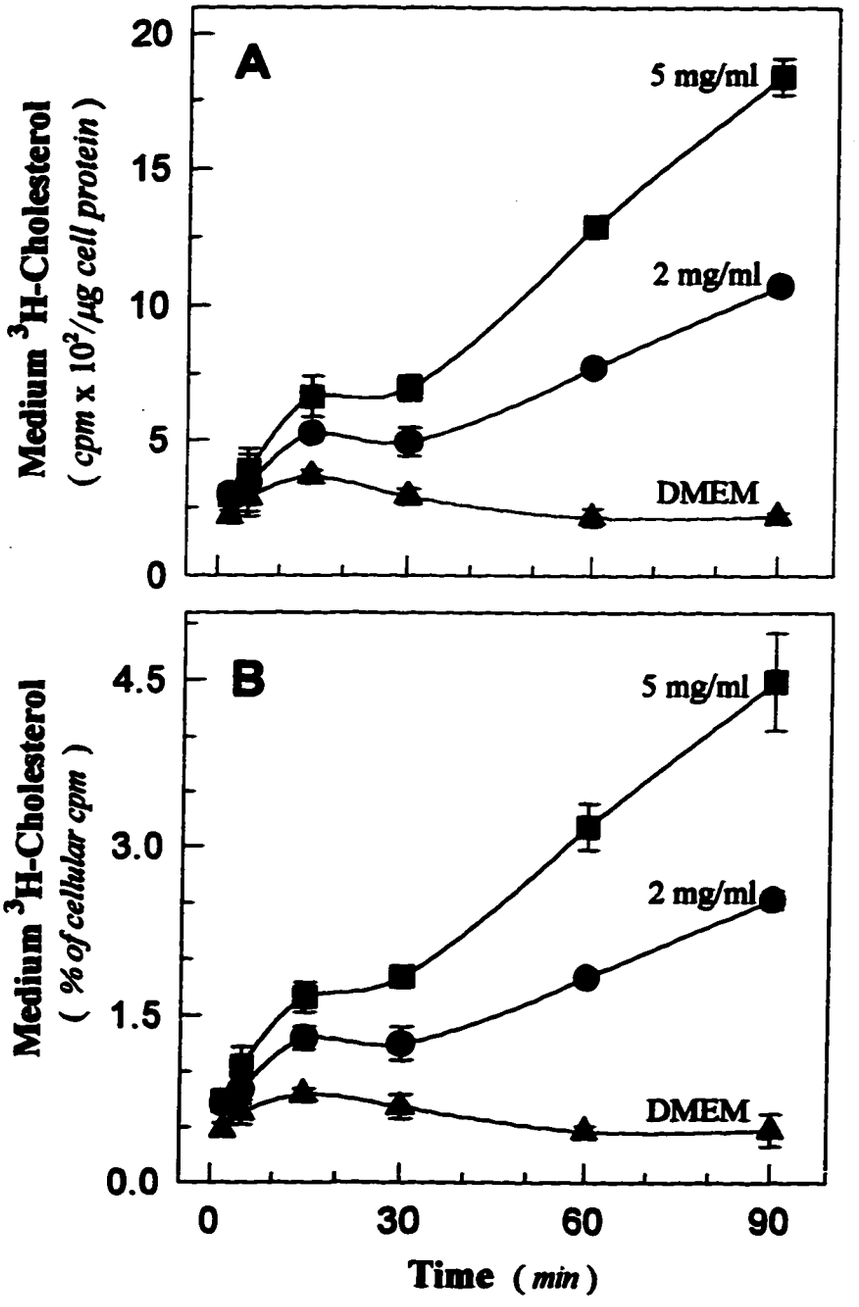

A
B
5 mg/ml
2 mg/ml
DMEM
Medium $^3$H-Cholesterol
( cpm x $10^2$/μg cell protein )
( % of cellular cpm )
Time ( min )
0
5
10
15
20
0.0
1.5
3.0
4.5
0
30
60
90

**Fig. 2-2.** ***Cellular Cholesterol Efflux as a Function of HSA Concentration***

Human skin fibroblasts were labelled as described in Fig. 2-1. After washing, cells were incubated with DMEM containing 0.5, 1, 2, 5 and 10 mg/ml of HSA. DMEM without HSA was used as control. Aliquots of medium were taken at 30, 60 and 90 min respectively for determination of radioactivity. Panel A shows the efflux expressed as medium radioactivity per μg cell protein. Panel B shows the efflux expressed as percentage of ($^3$H)-cholesterol in the medium relative to total cellular radioactivity. Each data point presented is the mean of 4 replicate samples and the error bar is the standard deviation.

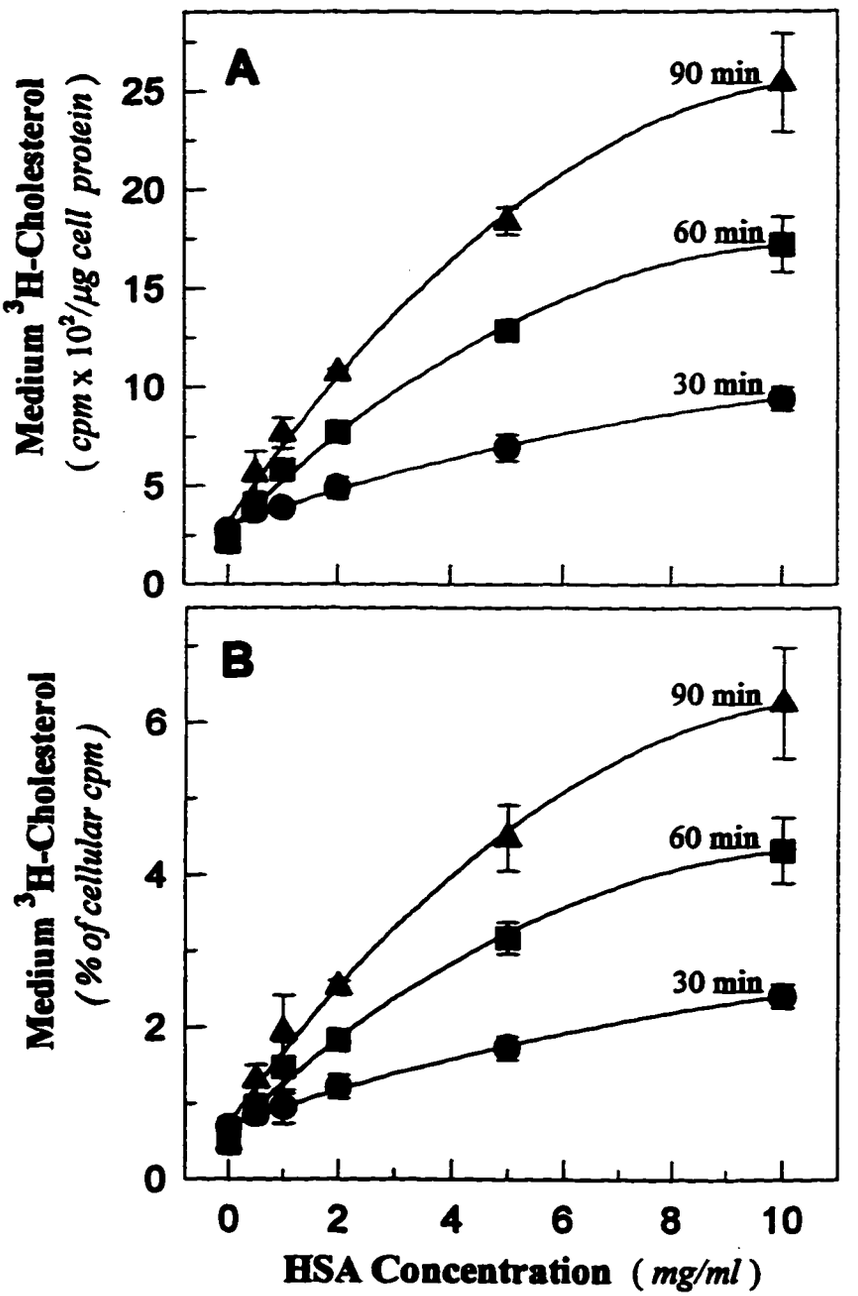

A
B
90 min
60 min
30 min
Medium $^3$H-Cholesterol
( cpm x $10^2$/μg cell protein )
Medium $^3$H-Cholesterol
( % of cellular cpm )
0
5
10
15
20
25
2
4
6
8
10
HSA Concentration ( mg/ml )

**Fig. 2-3.** ***Comparison of Cholesterol Efflux to HSA and to Reconstituted Discoidal LpA-I***

($^3$H)-Cholesterol labelled human skin fibroblasts were incubated with DMEM containing either or both 2 mg/ml of HSA and 45 μg protein/ml LpA-I. Cholesterol efflux was measured by taking medium aliquots at 60 min and 90 min respectively for radioactivity counting. The data are expressed as medium radioactivity per μg cell protein. T-test shows that the difference in efflux rates was statistically significant at 60 min ($p<0.05$) and 90 min of incubation ($p<0.01$) between HSA alone and HSA plus LpA-I, and at 90 min between LpA-I alone and HSA plus LpA-I ($p< 0.01$). Each data point presented is the mean of 4 replicate samples and the error bar is the standard deviation.

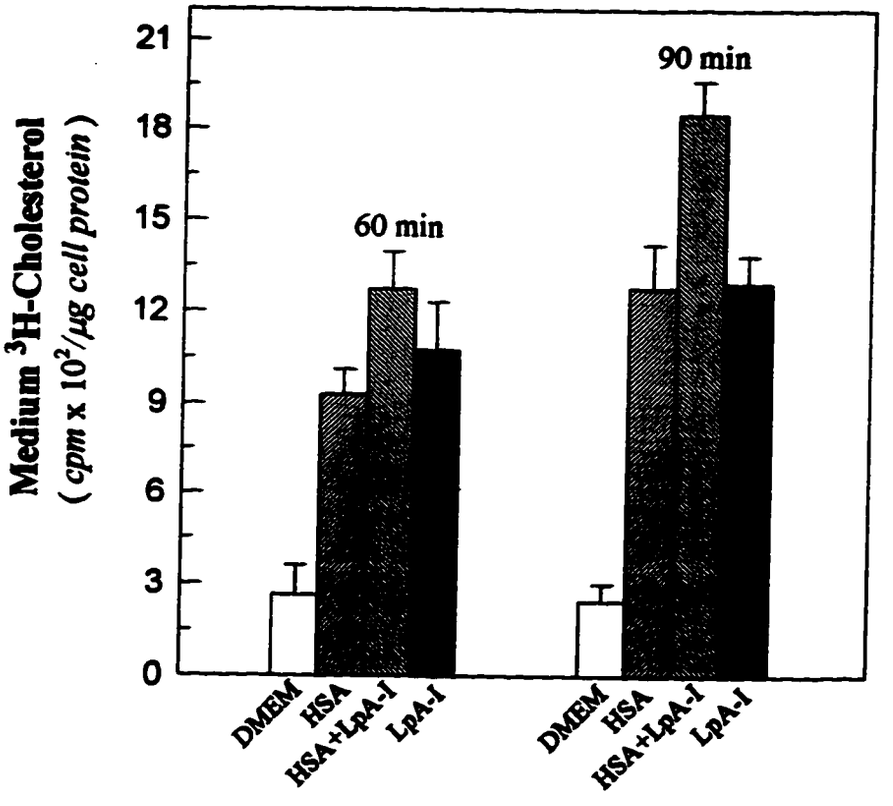

Medium $^3$H-Cholesterol
( cpm x $10^2$/μg cell protein )
21
18
15
12
9
6
3
0
60 min
90 min
DMEM
HSA
HSA+LpA-I
LpA-I
DMEM
HSA
HSA+LpA-I
LpA-I

**Fig. 2-4.** ***Comparison of the Effects of HSA, Gelatin and Ovalbumin on Cellular Cholesterol Efflux***

Human skin fibroblasts were labelled as described in Fig. 2-1. Cellular cholesterol efflux was studied in DMEM medium containing HSA, gelatin or ovalbumin at concentrations of 0.5, 1 or 2 mg/ml. DMEM alone was used as control (showed as the dotted line). Medium aliquots were taken at 60 min for radioactivity determination, and efflux were expressed as medium radioactivity per μg cell protein. Each data point presented is the mean of 4 replicate samples and the error bar is the standard deviation.

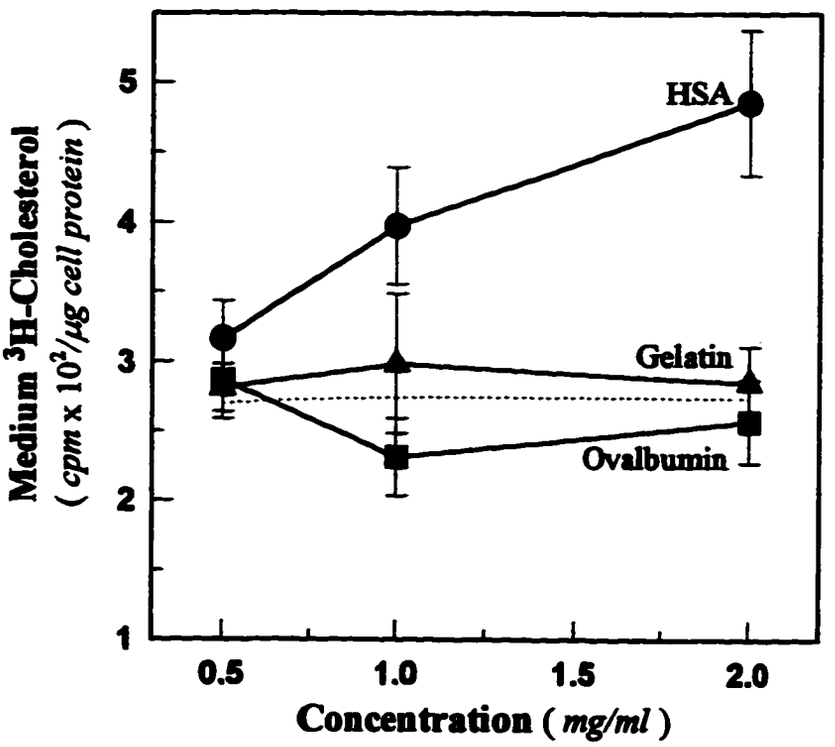
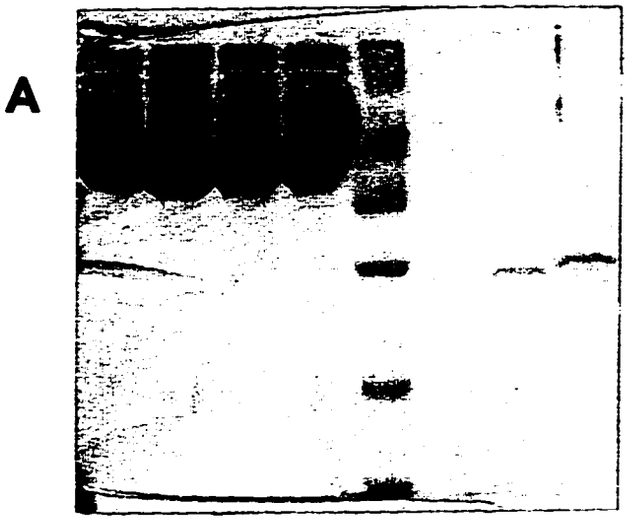

Medium $^3$H-Cholesterol
( cpm x $10^2$/μg cell protein )
5
4
3
2
1
HSA
Gelatin
Ovalbumin
0.5
1.0
1.5
2.0
Concentration ( mg/ml )

**Fig. 2-5.** ***Identification of ApoA-I in Different Commercial HSA Preparations***

Different HSA preparations were loaded at 50 μg/lane and electrophoresed on 4-20% native gradient PAGE gel. Panel A represents the protein stained with Coomassie blue. Lanes 1, 2, 3, and 4 are essentially fatty acid/globulin-free HSA, globulin-free HSA, essentially fatty acid-free HSA and regular fraction V HSA respectively. Lane 5 is the low molecular weight protein standard. Lane 7, 8 and 9 are purified lipid-free apoA-I loaded at 0.5, 1 and 1.5 μg/ml respectively. Panel B is the duplicate gel of Panel A, immunobloted with 4H1, a monoclonal antibody against the N-terminal 2-8 amino acid residues of apoA-I.


A
1 2 3 4 5 6 7 8

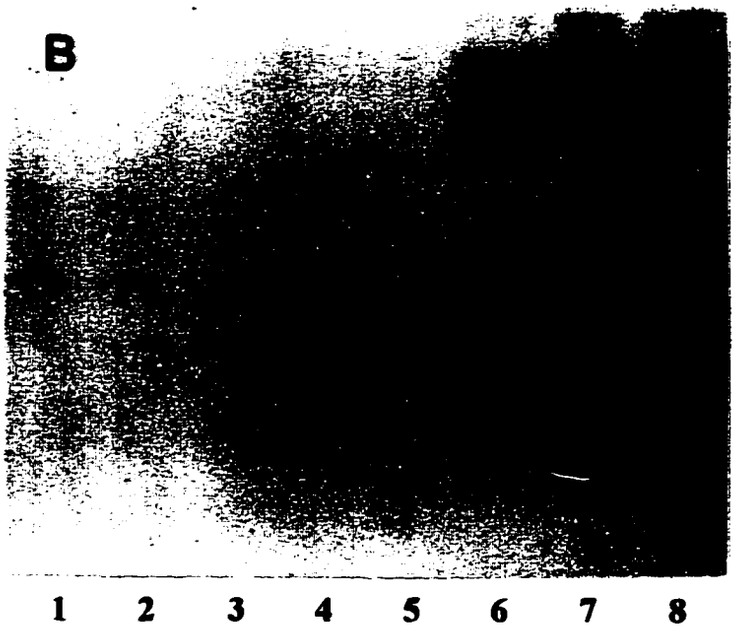

B
1 2 3 4 5 6 7 8

**Fig. 2-6.** ***Elution Profile of HSA-($^3$H)-Cholesterol Complexes***

Cholesterol was incorporated into HSA by sonication as described in Chapter 1. After passing through a 0.45 μm filter, the ($^3$H)-cholesterol labelled HSA was purified on a Superose 12 column running at a flow rate of 0.25 ml/min. Fractions collected (1.5 ml/fraction) are represented as filled circles for radioactivity, and as open circles for relative absorbance at 280 nm.

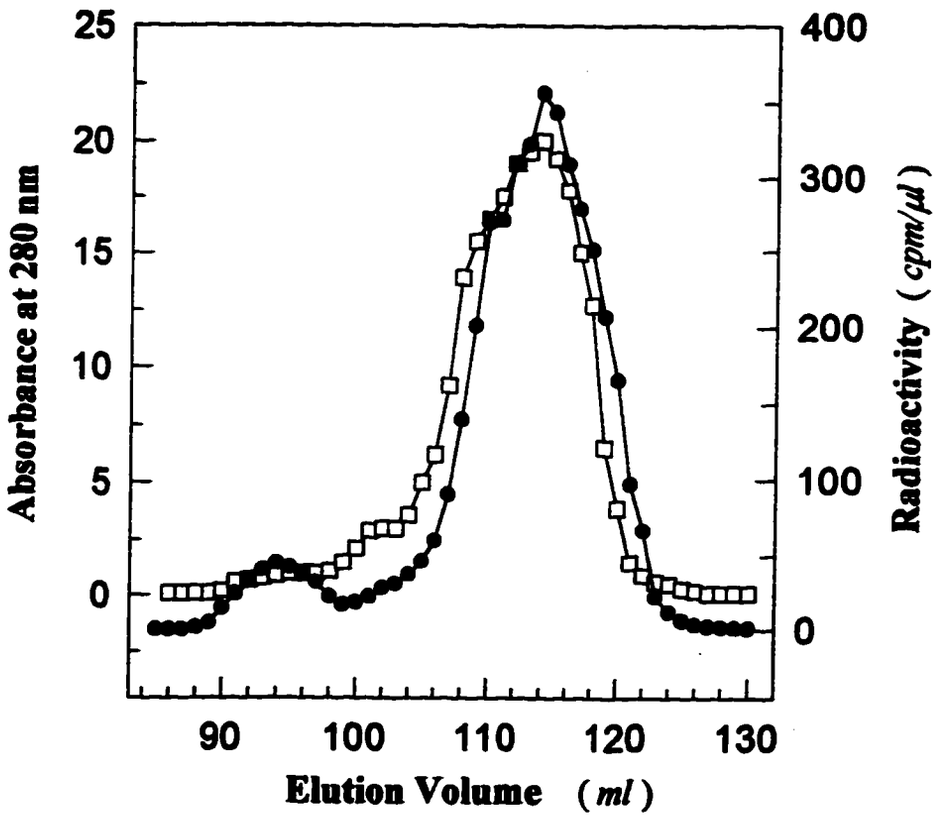

25
20
15
10
5
0
Absorbance at 280 nm
400
300
200
100
0
Radioactivity ( cpm/μl )
90
100
110
120
130
Elution Volume ( ml )

**Fig. 2-7.** ***Transfer of Cholesterol from HSA-($^3$H)-Cholesterol Complexes or from LpA-I-($^3$H)-Cholesterol Particles to Fibroblasts***

Fibroblasts were seeded and grown under the same conditions as in other efflux studies but without ($^3$H)-cholesterol label. The cells were then incubated with DMEM containing either HSA-($^3$H)-cholesterol complex ($4.81 \times 10^6$ cpm/μmole protein, $2.01 \times 10^8$ cpm/μmole cholesterol) at a concentration of 2 mg/ml, or 45 μg protein/ml discoidal reconstituted Lp2A-I pre-labelled with ($^3$H)-cholesterol ($9.64 \times 10^8$ cpm/μmole apoA-I or $3.34 \times 10^8$ cpm/μmole cholesterol). At the indicated time intervals, media were taken out, cells were washed twice with PBS in the presence of 2 mg/ml of BSA and twice with PBS alone. The washed cells were then lysed in 0.5 ml 0.1 N NaOH and aliquots were used for radioactivity counting. Panel A represents the percentage of ($^3$H)-cholesterol transferred to the cells. Panel B gives the calculated mass transfer of cholesterol to the cells. Each data point presented is the mean of 4 replicate samples and the error bar is the standard deviation.

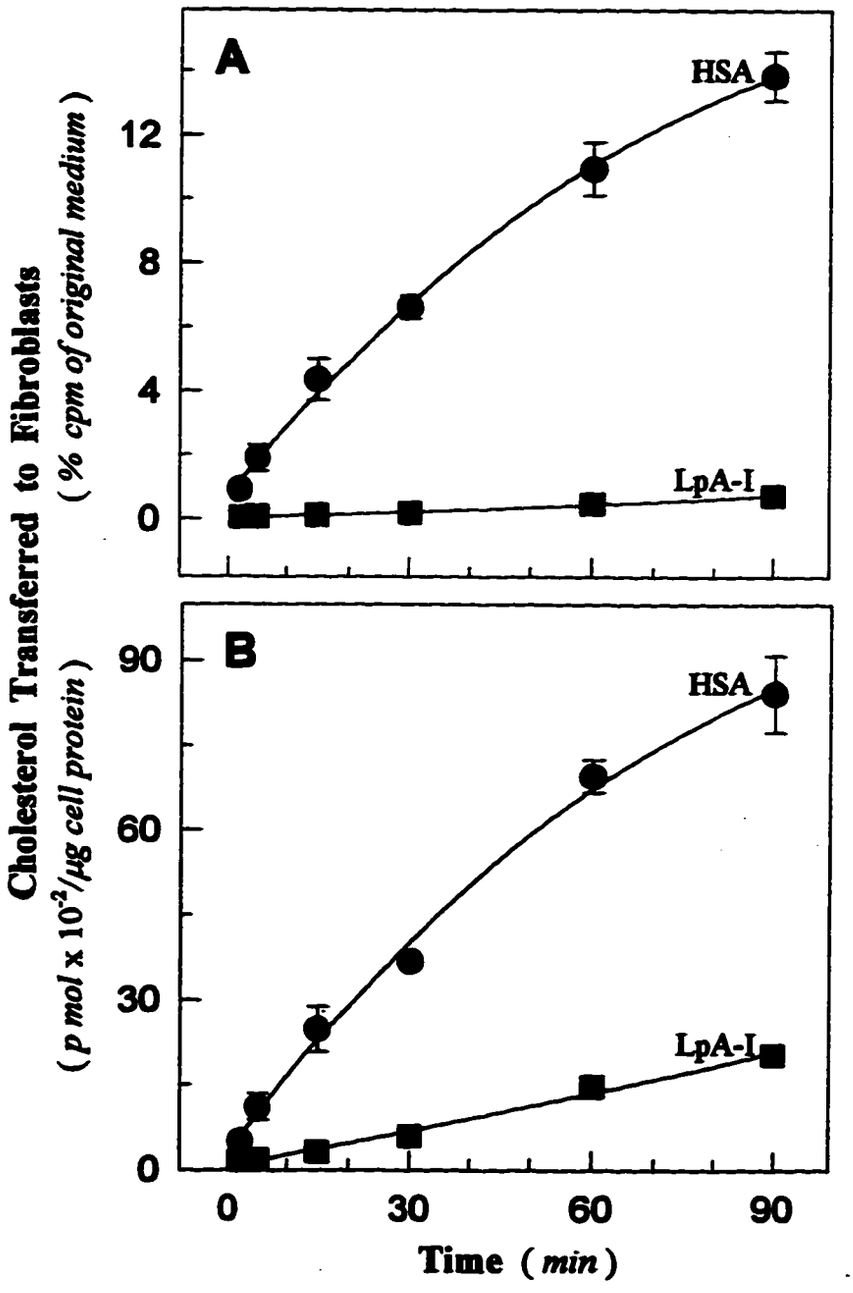

A
HSA
LpA-I
12
8
4
0
B
HSA
LpA-I
90
60
30
0
0
30
60
90
Time ( min )
Cholesterol Transferred to Fibroblasts
( % cpm of original medium )
( p mol x 10-2/μg cell protein )

**Fig. 2-8.** ***Transfer of Cell-Derived Cholesterol from HSA or LpA-I to Cells***

Human skin fibroblasts were labeled with 20 μCi/well of ($^3$H)-cholesterol and after washing were incubated with fresh DMEM medium containing either 2 mg/ml of HSA or 45 μg protein/ml of reconstituted discoidal Lp2A-I as described for Fig. 2-1. After 3 h of incubation, the media were taken out, centrifuged to remove any detached cells, and then transferred to other 12 well plates containing unlabelled and washed cell monolayers that had been incubated in the presence of 5% of FBS for 48 h. After 15 and 90 min of incubation respectively at 37°C, the media were taken out and the cells were washed and lysed with 0.1 NaOH. The radioactivity in both media and cell lysate were counted. The data are expressed as the percentage of radioactivity transferred to cells. Each data point presented is the mean of 4 replicate samples and the error bar is the standard deviation.

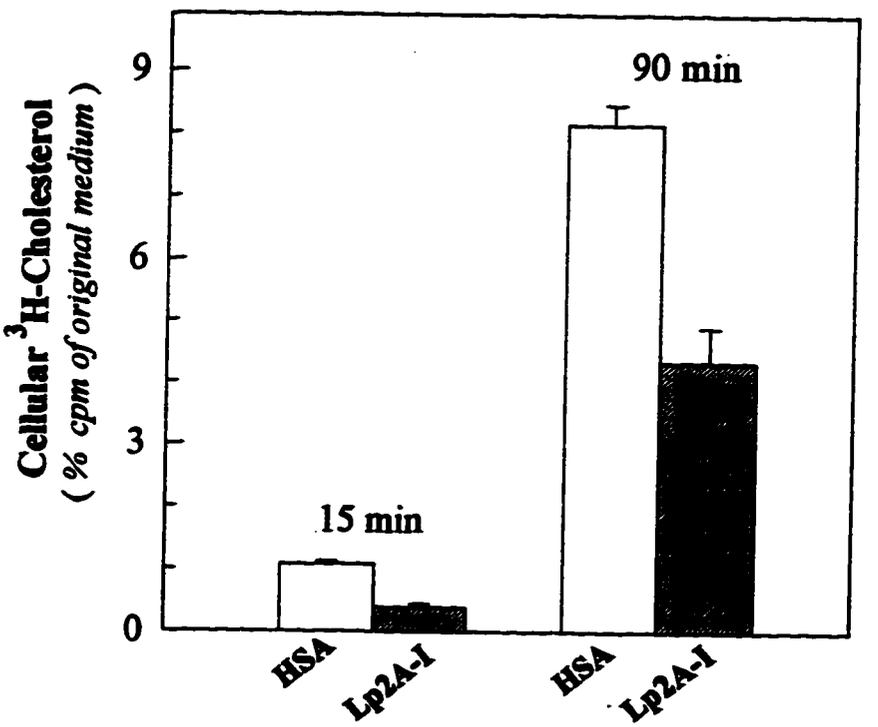
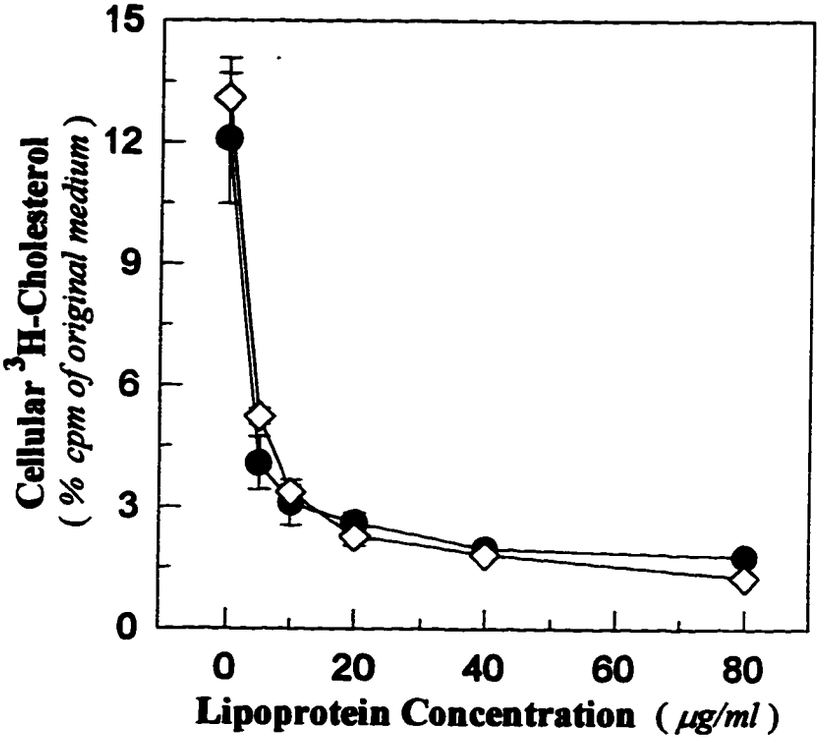

Cellular $^{3}$H-Cholesterol
( % cpm of original medium )
9
6
3
0
90 min
15 min
HSA
Lp2A-I
HSA
Lp2A-I

**Fig. 2-9.** ***Transfer of Cholesterol from HSA-($^3$H)-Cholesterol Complexes to Fibroblasts in the Presence of Reconstituted Lp2A-I or LDL***

Fibroblasts were treated as described for Fig. 2-7 except that the incubation medium contained 5, 10, 20, 40, and 80 μg protein/ml of reconstituted discoidal LpA-I or freshly separated LDL which were added to the HSA-($^3$H)-cholesterol complexes immediately before addition to the cells. For the incubation in the presence of LDL, this lipoprotein was first preincubated with anti apoB mAb (4G3 and 5E11) at 3 times molar excess, 37$^0$C for 1.5 h before added to the medium. Media were taken out at 90 min of incubation, and cells were washed twice with PBS in the presence of 2 mg/ml of BSA, and twice with PBS alone. The washed cells were then lysed in 0.5 ml of 0.1 N NaOH, from which 200 μl was taken for radioactivity counting. The open circles represent the percentage of ($^3$H)-cholesterol transferred to the cells in the presence of LDL, while the filled circles are the transfer of ($^3$H)-cholesterol in the presence of Lp2A-I (n=4, +/- SD).


15
12
9
6
3
0
0
20
40
60
80
Cellular $^{3}$H-Cholesterol
( % cpm of original medium )
Lipoprotein Concentration ( μg/ml )

# CHAPTER 3

## The Effect of the Apolipoprotein A-I and Surface Lipid Composition of Reconstituted Discoidal HDL on Cholesterol Efflux from Cultured Fibroblasts

## I. SUMMARY

Five series of reconstituted discoidal HDL (LpA-I) particles have been prepared and their constituents, apolipoprotein A-I (apoA-I), 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC), unesterified cholesterol (UC), phosphatidylinositol (PI), or sphingomyelin (SM) have been systematically varied to elucidate the relationship between HDL composition and cholesterol efflux from non-cholesterol loaded human skin fibroblasts. The physical properties, such as Stoke's diameters, α-helix contents, and surface potentials of these LpA-I have been measured and related to the ability of the LpA-I to accept cellular cholesterol. The results show that for LpA-I particles containing 2, 3 or 4 apoA-I per particle, Lp4A-I are the best acceptors of cellular cholesterol, followed by Lp3A-I and then Lp2A-I particles. Discoidal Lp2A-I with variations in POPC content, from 121 to 266 mol/particle, show no difference in their abilities to promote cholesterol

efflux. Similarly, inclusion of 7 and 15 moles of free cholesterol to Lp2A-I also does not affect their ability to accept cellular cholesterol. However, increasing the content of either PI or SM, up to 20 moles/particle, is associated with significantly increased abilities of the LpA-I to promote cholesterol efflux. The efflux of cellular cholesterol to discoidal LpA-I particles is independent of specific changes in apoA-I conformation and charge, but appears to be positively related to major changes in the size of the lipoprotein particles and more dependent on the ability of a HDL particle to adsorb and retain cholesterol molecules. Therefore, variations in the size of HDL and the concentrations of SM and PI within HDL would be expected to affect plasma cholesterol homeostasis.

# II INTRODUCTION

The removal of cholesterol from extrahepatic cells and further transport to the liver for catabolism, a process known as "reverse cholesterol transport" (RCT) (Glomset, 1968), has been suggested to be an important mechanism for the anti-atherogenetic function of HDL. The initiation of this process, the desorption of cholesterol from the plasma membrane of extrahepatic cells to HDL particles in extracellular compartments, has been reviewed as a limiting step in RCT (Rothblat et al., 1992, Johnson et al., 1991). It has been suggested that the transfer of cholesterol from cell plasma membrane to HDL operates mainly through an aqueous diffusion mechanism (Phillips et al., 1987, Johnson et al., 1991), which does not require the binding of HDL to specific membrane sites (Karlin et al., 1987, Slotte et al., 1987, Johnson et al., 1988, Mendel et al., 1988, Aviram et al., 1989). However, there is also another mechanism which is regulated and dependent on the translocation of cholesterol from intracellular pools to the plasma membrane (Brinton et al., 1986, Slotte et al., 1987, Aviram et al., 1989, Oram et al., 1991).

The great heterogeneity of HDL population in interstitial/lymph as well as in plasma makes it possible that different species of HDL may have distinct capacity to receive cellular cholesterol. An apoA-I containing pre β migrating HDL has been shown to be the first acceptor for cell-derived cholesterol (Castro &

Fielding, 1988), from where the cell-derived cholesterol proceeds further to pre $\beta_2$- then to pre $\beta_3$- and to $\alpha$–HDL for esterification (Francone et al., 1989, Francone & Fielding, 1990, Huang et al., 1993). Recently, $\gamma$-LpE, a subspecies of HDL containing apoE only, and LpA-IV, a lipoprotein containing only apoA-IV have been reported to be other preferential acceptors for cell-derived cholesterol (Huang et al., 1994, von Eckardstein et al., 1995).

Using reconstituted discoidal LpA-I particles, an earlier study from this laboratory has suggested that the apoA-I/phospholipid related size of LpA-I was an important contributor to the ability of LpA-I to accept cell-derived cholesterol (Agnani & Marcel, 1993). The larger reconstituted discoidal Lp4A-I have a higher capacity than the smaller Lp3A-I which have a higher capacity than Lp2A-I to accept cell-derived cholesterol from non-cholesterol loaded human skin fibroblasts. Similar results were also obtained by others in different cell lines and using longer incubation times (Jonas et al., 1994, Davidson et al., 1995a). However, in these early studies, reconstituted particles were used with more than one component being varied at a time, thus preventing a definitive analysis of the effect of size and content of individual constituents on efflux to lipoproteins. Here we have conducted a detailed time- and concentration-dependent study of efflux to discoidal lipoproteins with systematic variation of each component. We prepared

five series of reconstituted lipoproteins with varying components, such as apoA-I, PC, PI, SM and UC, within the range found in native HDL particles. The physico-chemical properties of these discoidal particles have been characterized and their avidity for binding cellular cholesterol has been analyzed.

# III. RESULTS

## 1. *Characterization of the homogeneity, composition and size of discoidal LpA-I particles*

The discoidal LpA-I particles prepared at 4°C from an initial POPC/apoA-I mixture of 120/1 (molar ratio) show three subspecies which can be re-isolated by size exclusion chromatography (Jonas et al., 1989, Nichols et al., 1987). The homogeneity of these discoidal LpA-I is confirmed by NDGGE. As summarized in Table 3-I, the isolated LpA-I particles contain 2, 3 and 4 apoA-I per particle respectively as determined by DMS cross-linking. Their molar ratios of POPC/apoA-I are 78/1, 111/1 and 131/1, and Stoke's diameters are 9.1, 12.7, and 17.1 nm respectively. It is apparent that both apoA-I and POPC molecules contribute to the size difference among these particles since LpA-I particles with more apoA-I have higher POPC levels. As noted by Jonas et al (1989), the determination of discoidal lipoprotein sizes by NDGGE does underestimate their size by about 10% compared to electron microscopy.

In contrast to the heterogeneous population of LpA-I particles generated at 4°C, dispersions of POPC and apoA-I with sodium cholate at 37°C produce homogenous particles when the POPC/apoA-I molar ratios of initial mixtures are kept within the optimal range from 40/1 to 140/1. Each of these particles contains

2 apoA-I and those prepared from the initial molar ratios of 60/1, 100/1 and 140/1 (POPC/apoA-I) are named D1, D2, and D3. The POPC/apoA-I final molar ratios of these LpA-I are 61/1, 100/1 and 133/1 respectively, demonstrating a greater than 95% incorporation of POPC into LpA-I. Varying the phospholipid content of Lp2A-I is associated with small changes in particle size, with the Stoke's diameters increased by approximately 5% following the increase of POPC content from 61/1 to 100/1 and to 133/1 (9.2, 9.5 and 9.7 nm respectively for D1, D2, and D3) (Table 3-II). When POPC/apoA-I reaches a molar ratio above 140/1, a larger subspecies of LpA-I appears migrating to a position on NDGGE corresponding to Lp3A-I, in agreement with a previous report (Davidson et al., 1995a). It is therefore, apparent that the apoA-I number is the key component for the total level of phospholipid incorporated in reconstituted LpA-I and their resulting sizes. Addition of 4 or 8 moles of free cholesterol does not affect either the homogeneity of Lp2A-I or the amount of POPC incorporated per particle. The presence of UC at the above mentioned ratios also slightly increases the Stoke's diameter of these Lp2A-I from 9.3 to 9.7 nm (Table 3-II), in agreement with a previous report (Sparks et al., 1993).

In this study we show that up to 30 moles of SM (15 moles/apoA-I) can be incorporated into reconstituted discoidal Lp2A-I particles without affecting their homogeneity. When the starting SM to POPC ratios are progressively increased

while maintaining a constant POPC concentration, the resulting LpA-I contains increasing amount of SM but reduced POPC (Table 3-I). This suggests that SM has, not only as reported earlier a high affinity for cholesterol (Lund-Katz et al., 1988; Yeagle and Young, 1986; Fugler et al., 1985), but may also compete with POPC to bind to apoA-I (Swaney, 1983). The increased total phospholipid content per particle with the increasing SM is accompanied by a slight increment in particle size, which increases from 9.4 to 10.1 nm as SM increases from 0 to 15.1 moles (Table 3-II). Discoidal Lp2A-I particles containing PI have also been prepared and characterized (Table 3-I and 3-II). The yield of incorporation of PI into LpA-I is about 70%, and the increase in PI content from zero to 2.8 and 5.6 moles per apoA-I results in a small non-significant decrease in particle size (10.1 versus 9.1 and 8.9 nm).

2. ***Effect of composition on the physical properties of discoidal LpA-I***

Variation in the composition of discoidal LpA-I is associated with changes in apoA-I conformation which are accompanied by variations in several representative physical parameters, such as the α-helix content, the electrophoretic migration and the surface potential. Since the predominant phospholipid components of LpA-I, such as PC and SM are un-charged, the change in the electrophoretic migration or surface potential of LpA-I accompanying a variation

in the content of these lipids will represent an alteration in the secondary and/or tertiary structure of apoA-I (Sparks et al., 1992a, b). All the reconstituted discoidal LpA-I particles prepared with the exception of those containing PI exhibit surface potentials corresponding to the pre β migration range on agarose gel (Table 3-II, Fig. 3-1). The surface potentials are negatively related to the apoA-I number, the POPC content, and SM content of these particles. At the ratio studied, the presence of free cholesterol does not affect significantly the particles' surface charge. As expected, a progressive increase in the surface potential of Lp2A-I containing PI is observed reflecting the negative charge of this phospholipid (Table 3-II and Fig. 3-1). The presence of 5.6 moles of PI per particle shifts the migration of the Lp2A-I particles from a position intermediate between pre β and α position to an α position (Fig. 3-1).

Although apoA-I, POPC and SM all appear to reduce the surface potentials of reconstituted discoidal LpA-I, they affect the structure of LpA-I differently. Circular dichroic measurements show that apoA-I on the Lp2A-I particles has an average α–helix content of 72%, while those on Lp3A-I and Lp4A-I are 58% and 56% respectively. Also, the increase in POPC content of LpA-I is accompanied by increases in α–helix content in contrast to the decreased surface potentials observed for the same particles. Addition of UC reduces the α–helix contents of

the lipid bound apoA-I, but no effect on surface potential is observed (Table 3-II). In LpA-I particles containing a constant level of UC, addition of 4.4 moles of SM significantly increases the α-helix content from 66% to 74%; however, further increase in SM causes a minor decrease in the α-helix content of LpA-I. The presence of PI does not affect the α-helix content of Lp2A-I, but as expected, significantly enhances the surface potential of the corresponding LpA-I particles (Table 3-II).

### 3. *Effect of LpA-I composition on cellular cholesterol efflux*

A previous study in this laboratory has shown that in a 5 min incubation with fibroblasts, Lp4A-I could promote more cholesterol efflux than Lp3A-I or Lp2A-I (Agnani and Marcel, 1993). Here, we have investigated in detail the time and concentration dependent efflux of cholesterol from fibroblasts to these defined and homogeneous discoidal particles. As showed in Fig. 2, when Lp2A-I, Lp3A-I or Lp4A-I at the same protein concentration (45 μg/ml) are incubated with $^{3}$H-cholesterol labelled, non-cholesterol-loaded fibroblasts, each of them has a similar ability to promote cholesterol efflux during an incubation period of 2 to 90 min. However, since these lipoproteins contain different number of apoA-I per particle, a more meaningful comparison of these particles requires that their protein concentration in the incubation media be adjusted to provide the same particle

concentration for each type of LpA-I. As shown in Fig. 3-3, when Lp2A-I, Lp3A-I or Lp4A-I are added to the incubation medium of fibroblasts at the same particle concentration, the efflux to Lp4A-I is significantly higher than that to Lp3A-I and to Lp2A-I. The difference in cellular cholesterol efflux is also statistically significant between Lp2A-I and Lp3A-I at 90 min of incubation ($p<0.05$). Concentration dependent cholesterol efflux to Lp2A-I or Lp4A-I has also been studied. Again, at the same protein concentration of LpA-I in medium, similar efflux rates to Lp2A-I and Lp4A-I are observed (Fig. 3-4A). However, at the same particle concentration in the medium, cholesterol efflux to Lp4A-I is again significantly higher than that to Lp2A-I (Fig. 3-4B). As observed with native HDL (Johnson et al., 1986, 1990), the efflux promoted by these particles does not saturate up to 200 μg protein/ml.

Since LpA-I particles with different number of apoA-I also vary in POPC content, we have studied cellular cholesterol efflux to LpA-I with constant 2 apoA-I per particle but varying POPC levels to determine whether the POPC/apoA-I ratio in itself contributes to the ability of LpA-I to receive cellular cholesterol. As shown in Fig. 3-5, varying the phospholipid content of Lp2A-I (D1, D2 and D3) does not significantly alter their abilities to promote cellular cholesterol efflux when they are added to the medium at the same concentration of 45 μg protein/ml. Therefore, taken together with previous experiments, these results suggest that

apoA-I number, which is the major determinant of both the level of phospholipid incorporation and the size of LpA-I, is the most important component dictating the ability of LpA-I to promote cellular cholesterol efflux to discoidal LpA-I.

The incorporation of cholesterol into LpA-I is limited by the reconstitution technique to the maximal level of 15.4 moles per particle. As showed in Fig. 3-6, addition of 6.8 or 15.4 moles of UC into Lp2A-I particles does not affect their ability to promote cellular cholesterol efflux.

The time-dependent efflux of UC from non-cholesterol-loaded human skin fibroblasts to reconstituted discoidal LpA-I exhibits a bi-phasic pattern (Fig. 3-2, 3-5, 3-6 and 3-7). An early rapid efflux phase is observed during the first 15 min of incubation, followed by a plateau up to about 30 min, and a second linear phase starts from about 30 to 60 min. This bi-phasic efflux may represent the transition of the cholesterol efflux process from the pool represented by only cell plasma membrane to that also including mobilization of cholesterol from intracellular pools (Mahlberg and Rothblat, 1992).

In contrast to the efflux to reconstituted discoidal Lp2A-I containing varying POPC or UC content, the addition of only 2.8 moles of PI per apoA-I (PI1) does translate into a significant increase in cholesterol efflux (Fig. 3-7). This enhancement is rapid, occurs in the first phase of cholesterol efflux, and remains constant during the lag period and the second phase. It may represent a specific

ability of this PI containing particle to release UC from the plasma membrane. Incorporation of more PI into Lp2A-I has no further effect on efflux (Fig. 3-7).

The time dependent efflux to SM-containing Lp2A-I is increased significantly with the incorporation of 4.4 or 8.2 moles of SM per apoA-I, and appears to plateau at the same level (Fig. 3-8). In contrast to the efflux to PI-containing Lp2A-I, the increase in efflux to SM-containing Lp2A-I is greater in the second phase than that in the initial phase of efflux.

As shown in Fig. 3-9, we have observed several relationships between cholesterol efflux and the physico-chemical parameters of LpA-I containing 2-4 molecules of apoA-I. Cellular cholesterol efflux is correlated negatively to the surface potentials (Fig. 3-9A-1) and α–helix contents of these particles (Fig. 3-9B-1), and positively correlated to the Stoke's diameters of these particles (Fig. 3-9C-1). In contrast, no such relationships could be observed in Lp2A-I with varying POPC and UC levels. The changes of surface potential and α-helicity of Lp2A-I with varying POPC or varying UC do not result in corresponding alteration in cholesterol efflux (Fig 3-9A-1, 3-9B-1). Small changes in the Stoke's diameters of these particles are also not sufficient to elicit changes in cholesterol efflux (Fig 3-9C-1). Therefore, the efflux of cellular cholesterol to discoidal LpA-I particles is independent of their phospholipid or cholesterol contents, but appears associated with both the number of apoA-I molecules per particle, the accompanying change

in particle size. The enhanced abilities of Lp2A-I particles containing PI or SM in releasing cellular cholesterol are obviously not due to the alteration of any measured physico-chemical parameters (Fig. 3-9A-2, 3-9B-2, and 3-9C-2). Although enhanced surface potential appears to be related to the enhanced efflux in PI-containing Lp2A-I, further addition of PI up to 27 moles per particle does not result in corresponding enhancement in efflux (data not shown).

# IV. DISCUSSION

Discoidal HDL particles are thought to be a subspecies of HDL that are present in interstitial fluid/peripheral lymph as well as in the plasma of humans (Reichl et al., 1985, 1990) and animals (Sloop et al., 1983a, 1983b, Dory et al., 1985, Forte et al., 1983, Lefevre et al., 1988). Increases in plasma and interstitial fluid discoidal HDL appear to accompany several alterations of cholesterol metabolism, such as in LCAT deficiency (Jonas et al., 1993, Ohta et al., 1994), in the hypercholesterolemic and hypothyroid state (Sloop et al., 1983a), and in normal animals fed a high cholesterol chow (Dory et al., 1985). Like other HDL, discoidal HDL represent a group of heterogeneous particles differing in composition and size. Larger discoidal LpA-I particles containing several apoA-I molecules have been identified as the nascent HDL secreted by the liver (McCall et al., 1988), in the incubation of lipid-free apoA-I with non-transfected CHO-C19 cells (Forte et al., 1993), and in interstitial fluid/peripheral lymph (Reichl et al., 1990, Sloop et al., 1987, Lefevre et al., 1988). Their relatively high concentration in interstitial fluid/peripheral lymph, as compared to plasma (Sloop et al., 1983a, 1983b, Forte et al., 1983, Reichl et al., 1985, Dory et al., 1985) suggests that these particles may have a greater chance to interact with extrahepatic cells, and could play an important function in the efflux of cellular cholesterol, thus explaining their cholesterol enrichment in certain conditions as reviewed above.

In this investigation we have characterized the effect of variations in discoidal LpA-I composition on the ability of these lipoproteins to receive cholesterol from fibroblasts. These studies are designed to measure and compare the rates of cholesterol efflux after short term incubations (< 90 min), thus attempts to elucidate initial rates of cholesterol transfer between cells and intact LpA-I particles. It should be noted that as with all series of LpA-I, no particle fusion or major structural rearrangement of the LpA-I particles was evident after the efflux incubation. Whereas, in the more common long term (6-48 h) transfer studies of others (Davidson et al., 1995a, Jonas et al., 1994, Oram et al., 1991), it is possible that other factors that may be secreted or generated by the cultured cells during the long incubations alter lipoprotein composition and structure and subsequently modify lipoprotein and lipid equilibration.

In short term incubation studies with discoidal LpA-I, we have routinely observed a unique two phase pattern of cholesterol efflux, with the exception of efflux to SM-containing Lp2A-I. This bi-phasic profile appears to be a characteristic of human skin fibroblasts and is not evident in other studies with smooth muscle cells or endothelial cells (Marcel et al., unpublished observation). The rapid first phase occurs within the first 15 minutes, and is in general, independent of the presence or type of acceptors. In this phase, the rate of cholesterol efflux, with or without an acceptor lipoprotein particle, are similar

within the initial period (with the exception of PI-containing LpA-I). This suggests that the initial rate may depend mostly on the inherent ability of the cell plasma membrane to release cholesterol into medium, which does not appear to be affected by the absence or presence of different kinds of acceptors. The second phase, which is linear and starts between 30 and 60 min, appears to represent mostly the collision properties and/or ability of the acceptor lipoprotein to bind and retain cholesterol molecules. It is mainly during this second linear phase that we could demonstrate the most significant differences in cholesterol adsorption capacity between the different acceptor lipoproteins. The enhanced ability of specific LpA-I particles to accept cholesterol molecules in this study could reflect modified collision properties of the LpA-I or enhanced ability of the particles to bind and retain cholesterol.

This investigation has elucidated the abilities of discoidal Lp2A-I, Lp3A-I and Lp4A-I to promote cholesterol efflux and confirmed our earlier observations (Agnani and Marcel, 1993). When efflux rates are expressed on a per particle basis, it is evident that the larger Lp4A-I have the highest ability to promote cholesterol efflux from non-cholesterol loaded fibroblasts. Lp2A-I are the poorest acceptors of cellular cholesterol and Lp3A-I are somewhat intermediate between Lp4A-I and Lp2A-I. This conclusion is consistent with the data from an earlier report showing that, on a per particle basis, larger reconstituted discoidal Lp3A-I

(10.8 nm) and Lp2A-I (9.6 nm) have a higher ability to promote cholesterol efflux from adipocytes than do small Lp2A-I (7.8 nm). However, the authors of this report concluded that smaller particles had the higher ability to accept cholesterol, based on efflux rates that were corrected to a constant phospholipid concentration (Jonas et al., 1994). This differential interpretation reflects a common problem encountered when comparing different cholesterol efflux protocols. Indeed, our results also appear to be inconsistent with another recent report (Davidson et al., 1995a) which suggests that Lp2A-I with high POPC content and larger Stoke's diameters promote a greater cellular cholesterol efflux than smaller Lp2A-I particles. While in this study comparisons were made at the same particle concentration, the primary difference is that a longer efflux period (1-6 h) was used rather than our short period of efflux (2 to 90 min). Thus short term studies suggest that variation in the phospholipid content of LpA-I do not affect the ability of the lipoprotein to accept cholesterol. This observation may indicate that variations in the amount of phospholipid in a discoidal Lp2A-I particle has no effect on the ability of the lipoprotein to adsorb and/or retain cholesterol molecules. Neither hypothesis could be confirmed without simultaneously measuring the rate of cholesterol adsorption and desorption from the LpA-I surface. It is possible that the constant rates of efflux observed when POPC contents of LpA-I are varied may be a function of concomitant changes in

adsorption and desorption of cholesterol. This is consistent with what has been observed in transfers of cholesterol between LDL and LpA-I (Meng et al., 1995), which showed that increasing POPC/apoA-I ratio in similar discoidal LpA-I particles increases both the ability of the lipoprotein to accept as well as to donate cholesterol molecules to LDL.

Previous studies have suggested that a concentration gradient of cholesterol, usually represented by the UC/phospholipid ratio of the cell plasma membrane and HDL, determines the direction of the cholesterol exchange (Phillips et al., 1987). Since this UC/PC ratio is relatively constant for a given cell species; the ratio of UC/PC in HDL may be critical to whether HDL acts as either a donor or an acceptor of cholesterol. Experiments were performed to characterize the specific effect of UC on the ability of HDL to accept cellular cholesterol and it was shown that the incorporation of up to 16 molecules of UC into an Lp2A-I has no effect on cholesterol efflux. This may indicate that cholesterol has no effect on the ability of HDL to accept cholesterol or that this lipid simultaneously modifies both the adsorption and desorption of cholesterol. In transfers between LDL and LpA-I, we showed that increases in cholesterol in an LpA-I particle also affected the adsorption and desorption of cholesterol (Meng et al., 1995). In addition, inclusion of a high level of cholesterol also appeared to modify the kinetics of cholesterol transfer between LpA-I and LDL, from being independent of acceptor

concentration to a first order reaction that is dependent of LpA-I concentration. This suggested that changes in LpA-I cholesterol content may affect the rate of cholesterol exchange by changing the frequency of collisions between LDL and LpA-I particles. Since this same compositional modification had no effect on the efflux of cellular cholesterol, it follows that cholesterol exchange between cells and HDL particles may be less sensitive to the collision properties of discoidal LpA-I particles.

In HDL, SM and PI represent about 10% and 4% of total HDL phospholipid mass and as such are phospholipids secondary only to PC in abundance (Skipski, 1972). SM has been reported to have a higher affinity toward cholesterol in small unilamellar vesicles than other phospholipids (Lund-Katz et al., 1988, Yeagle and Young, 1986, Fugler et al., 1985). A positive subcellular co-distribution of SM with cholesterol has been well demonstrated in intact cells (Lange et al., 1989, Wattenberg and Silbert, 1983, van Blittersuijk et al., 1987). The important function of SM in the maintenance of cellular cholesterol homeostasis has become evident in the past decade. For example, addition of exogenous SM to cultured cells resulted in a marked increase of cholesterol biosynthesis and a reduction of LDL binding and degradation (Gatt and Bierman, 1980). Depletion of SM content of cell plasma membrane by SMase stimulates an extensive redistribution of cholesterol between plasma membrane and intracellular

pools (Slotte et al., 1989, 1990; Pörn et al., 1990, 1991). Since the addition of as little as 4 moles of SM per mole apoA-I significantly increases the ability of Lp2A-I to accept cellular cholesterol, it is assumed that the high ability of SM to associate with cholesterol allows the particle to bind and retain more cholesterol molecules.

Increased cholesterol efflux to particles containing SM appears to be specifically related to SM content because variation of either POPC or cholesterol contents in the same Lp2A-I has no effect on cholesterol efflux. The effect plateaus at about 16 moles of SM per particle where cholesterol efflux is nearly doubled as compared to the control (Fig. 3-8). This observation appears consistent with a previous study, which also reported elevated cholesterol efflux rates (when corrected per LpA-I particle) from both mouse L-cell fibroblasts and rat Fu5AH hepatoma cells to discoidal LpA-I particles prepared from SM (Davidson et al., 1995b). In an earlier study, cholesterol efflux from human skin fibroblasts has been shown to correlate positively with the SM content of a series of sonicated SM-liposomes (Stein et al., 1988). In addition, pre $\beta_1$-HDL, a subspecies of HDL that has been shown to have higher ability to accept cellular cholesterol may also contain higher levels of SM than other HDL subclasses (Fielding and Fielding, 1995a). The increased efflux-promoting ability of SM-containing Lp2A-I may be due to the influence of this phospholipid on the packing of the phospholipid

monolayer of HDL. SM may create packing defects in the surface of an HDL particle much like apoA-I is thought to (Davidson et al, 1995b, Letizia and Phillips, 1991). If interfacial packing defects promote the adsorption of cholesterol molecules, as proposed by Phillips and colleagues, SM may stimulate cholesterol efflux by actually creating spaces for incoming cholesterol molecules. Increasing the number of molecules of apoA-I on the surface of the discoidal particle (eg. in Lp3A-I and Lp4A-I) may similarly increase surface packing defects and thereby stimulate cholesterol adsorption.

Although our results indicate that introduction of PI into Lp2A-I significantly stimulates cholesterol efflux to these particles, the enhanced surface charge due to the phospholipid does not appear to be associated with this phenomena. We have observed no consistent relationship between particle surface potentials and the rates of cholesterol efflux for all series of LpA-I particles. The effect of PI on cholesterol transfers could thus be indirect and may result from a combined effect of charge and surface lipid packing, and may affect interaction with the cell membrane. While little is known about the effect of PI on lipid packing in the surface of a lipoprotein, it appears possible that this lipid, much like phosphatidylserine, may have a propensity to segregate into local charged regions in the presence of divalent cations (Paphadjopoulos et al., 1978). These regions may form crystalline nucleation points that would be expected to disrupt the

surface packing and may actually promote fusion with the surface lipids of other lipoproteins and/or cells. Increased negative charges at the surface of an HDL particle would also be expected to affect and perhaps repel negative charged residues in apoA-I. In this respect, PI may modify apoA-I conformation in a manner that may indirectly affect surface lipid packing and the ability of the LpA-I particle to adsorb cholesterol molecules.

We have previously shown that changes in discoidal LpA-I composition can have major effects on the ability of these lipoproteins to receive cholesterol from LDL particles (Meng et al., 1995). The study suggested that variations in the composition of LpA-I particles affect interlipoprotein cholesterol movement in a manner that is not consistent with a mechanism based purely upon aqueous diffusion of cholesterol (Phillips et al, 1987, Johnson et al., 1991). There is now mounting evidence that cholesterol exchange occurs by a more complex process that involves the desorption of UC from the plasma membrane, followed by collision with and incorporation into the acceptor particle (Thomas and Poznansky, 1988, Steck et al., 1988, Davidson et al., 1995a). Investigations by Fielding and colleagues (Miida et al., 1990) have further shown that cholesterol transfers between lipoproteins and between lipoproteins and cells may be regulated very differently. Evidence from the present and previous studies in this laboratory are in agreement with these observations and suggest that while factors that affect

the frequency of collisions between desorbed cholesterol and acceptor lipoproteins may govern interlipoprotein cholesterol transfers (Meng et al., 1995), cellular cholesterol efflux may be more dependent on factors that affect the ability of an HDL particle to bind and retain this lipid.

# V. CONCLUSION

We have shown in this study that when the number molecules of apoA-I, SM or PI increases in a discoidal LpA-I particle, these constituents can affect the ability of these lipoproteins to adsorb and retain cholesterol molecules from cultured human skin fibroblasts; in contrast to the increase of the number of POPC and UC. Therefore, the concentrations of apoA-I, SM and PI in various classes of discoidal lipoproteins may play a role in governing lipid transfer processes and if modified in hyperlipidemic subjects, they may significantly affect plasma cholesterol homeostasis. It is of interest that the efflux of cellular cholesterol to discoidal LpA-I particles does not appear to be affected by specific changes in apoA-I conformation and particle surface charge, but does appear to be related to major changes in the size of the lipoprotein particle.

**TABLE 3-I**

*Composition Analysis of Reconstituted Discoidal LpA-I*[a]

| LpA-I | Composition Molar Ratio (initial)[b] | Composition Molar Ratio (final)[c] | mol/LpA-I apoA-I[d] | mol/LpA-I T-PL[e] |
|---|---|---|---|---|
| Variation in apoA-I (POPC:ApoA-I) | | | | |
| Lp2A-I | 120:1 | 78:1 | 2 | 155 |
| Lp3A-I | 120:1 | 111:1 | 3 | 332 |
| Lp4A-I | 120:1 | 131:1 | 4 | 522 |
| Variation in phosphatidylcholine (POPC:ApoA-I) | | | | |
| D1 | 60:1 | 61:1 | 2 | 122 |
| D2 | 100:1 | 100:1 | 2 | 199 |
| D3 | 140:1 | 133:1 | 2 | 267 |
| Variation in free cholesterol (POPC:ApoA-I:UC) | | | | |
| C1 | 80:1:0 | 77:1:0 | 2 | 144 |
| C2 | 80:1:4 | 77:1:3.4 | 2 | 154 |
| C3 | 80:1:8 | 76:1:7.7 | 2 | 152 |
| Variation in sphingomyelin (POPC:ApoA-I:UC:SM) | | | | |
| SM1 | 80:1:4:5 | 72:1:3.6:4.4 | 2 | 76.4 |
| SM2 | 80:1:4:10 | 67:1:3.4:8.2 | 2 | 75.2 |
| SM3 | 80:1:4:20 | 58:1:3.5:15.1 | 2 | 73.1 |
| Variation in phosphatidylinositol (POPC:ApoA-I:UC:PI) | | | | |
| PI1 | 80:1:4:4 | 72:1:3.6:2.8 | 2 | 74.8 |
| PI2 | 80:1:4:8 | 67:1:3.4:5.6 | 2 | 72.6 |

a. Results are the averages of at least three preparations.

b. Molar ratios of initial mixtures for the preparations of reconstituted LpA-I.

c. Molar ratios of reconstituted LpA-I after re-isolation. The intersample variations are <5%.

d. Determined by protein cross-linking with DMS and subsquent SDS/PAGE.

e. Total phospholipid content of each LpA-I particle.

**TABLE 3-II**

*Characterization of Reconstituted Discoidal LpA-I*[a]

| LpA-I | Size (nm)[b] | α-Helix (%)[c] | Surf. Potential (-mV)[d] |
|---|---|---|---|
| Variation in apoA-I | | | |
| Lp2A-I | 9.1 | 72 | 6.5 |
| Lp3A-I | 12.7 | 58 | 5.5 |
| Lp4A-I | 17.1 | 56 | 4.9 |
| Variation in phosphatidylcholine | | | |
| D1 | 9.2 | 69 | 7.5 |
| D2 | 9.5 | 75 | 6.6 |
| D3 | 9.7 | 77 | 6.2 |
| Variation in free cholesterol | | | |
| C1 | 9.3 | 74 | 6.9 |
| C2 | 9.4 | 66 | 6.7 |
| C3 | 9.7 | 65 | 6.8 |
| Variation in sphingomyelin | | | |
| SM1 | 9.5 | 74 | 7.1 |
| SM2 | 9.9 | 70 | 6.6 |
| SM3 | 10.1 | 72 | 6.6 |
| Variation in phosphatidylinositol | | | |
| PI1 | 9.1 | 67 | 9.6 |
| PI2 | 8.9 | 68 | 11.6 |

a. Results are the averages of at least three preparations.

b. Stoke's diameters of LpA-I from NDGGE (±0.5 nm).

c. Determined from molar ellipticities at 222 nm in CD spectra (±4% SD).

d. Calculated from the electrophoretic migration of LpA-I on agarose gel (±0.2 SD).

**Fig. 3-1.** ***Electrophoretic Mobilities of Reconstituted Discoidal LpA-I***

Reconstituted discoidal LpA-I particles were electrophoresed on 0.6% agarose gel at 100 V for 30 min. The electrophoretic mobilities were calculated from the migration distance of these particles as previously described (Sparks et al., 1992). The standard pre β and α-migration positions were calculated from the migration distance of the corresponding lipoproteins from fresh plasma electrophoresed in the same conditions. Each data point presented is the mean of 4 or more replicate samples and the error bar is the standard deviation.

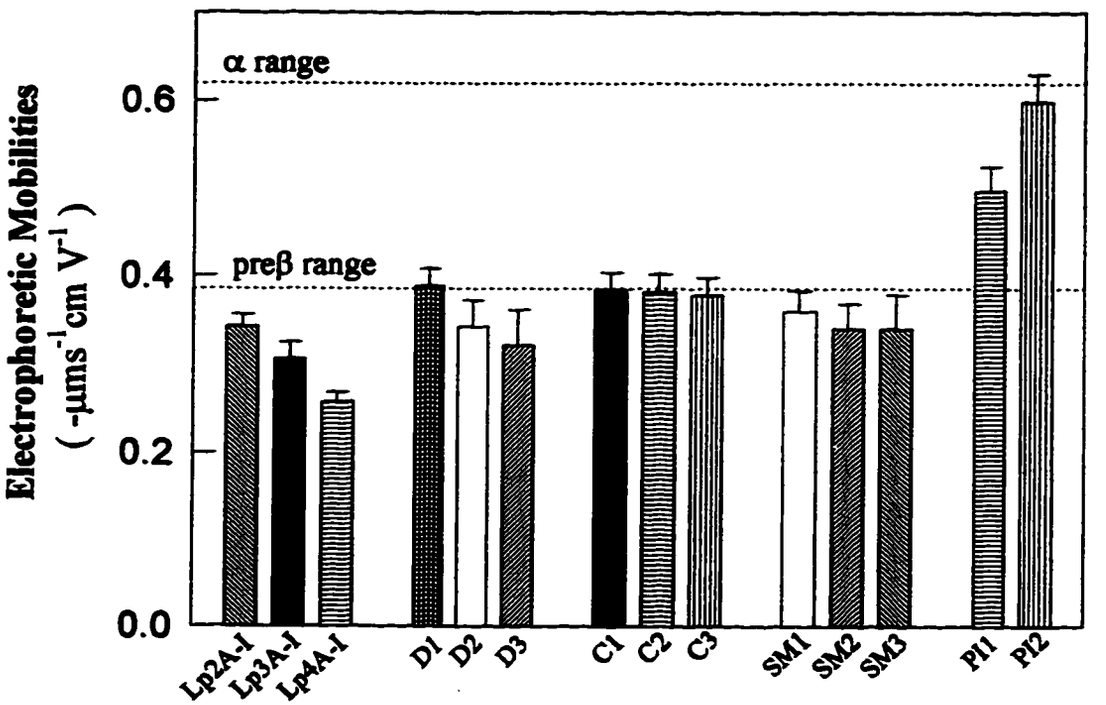

α range
preβ range
Electrophoretic Mobilities
(-μms-1cm V-1)
0.6
0.4
0.2
0.0
Lp2A-I
Lp3A-I
Lp4A-I
D1
D2
D3
C1
C2
C3
SM1
SM2
SM3
PI1
PI2

**Fig. 3-2.** ***Cholesterol Efflux to LpA-I with Varying ApoA-I Number Added to the Medium at the Same Protein Concentration***

Fibroblasts were seeded in 12 well plate at a density of 5.5 x $10^4$cells/well and grown at 37°C in the presence of 10% FBS for 48h to reach 70% confluence. After washing, the cell monolayers were labelled by incubation in a medium containing 1,2n-$^3$H-cholesterol (50 μCi/ml) and 5% FBS for 48 h. For the efflux study, the cells were washed and then incubated with DMEM containing Lp2A-I, Lp3A-I or Lp4A-I at a concentration of 45 μg protein/ml. Aliquots of medium were taken at the indicated times between 2 and 90 min for the determinations of radioactivity. Efflux is expressed as medium cpm /μg cell protein. Each data point presented is the mean of 4 replicate samples and the error bar is the standard deviation.

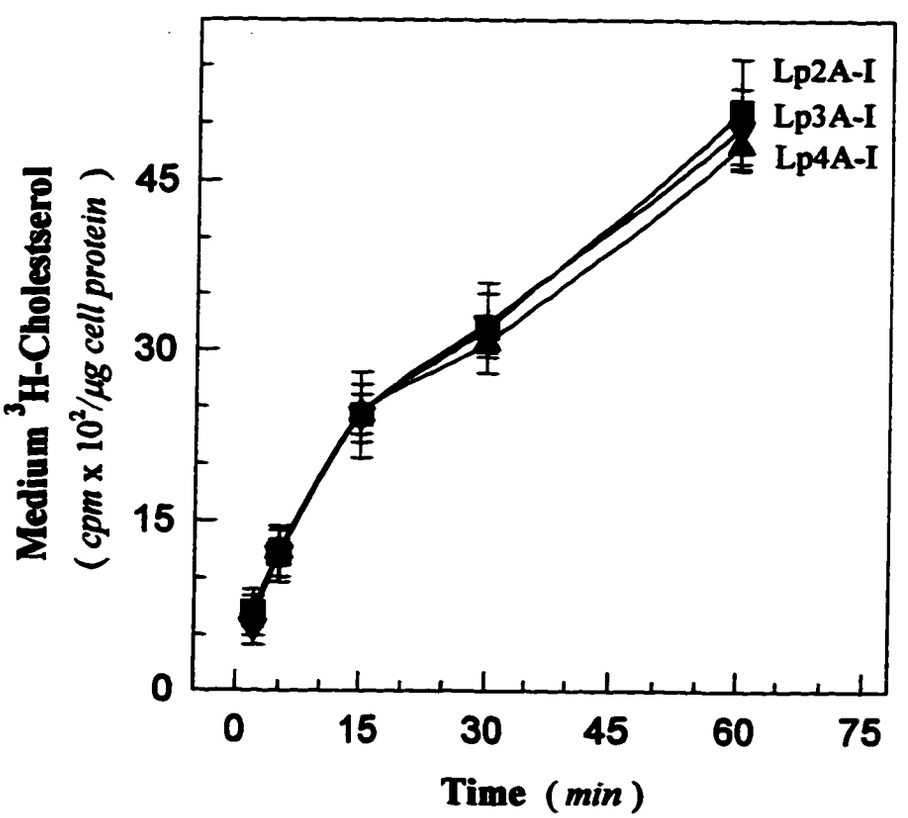

Lp2A-I
Lp3A-I
Lp4A-I
Medium $^3$H-Cholestserol
( cpm x $10^2$/μg cell protein )
0
15
30
45
0
15
30
45
60
75
Time ( min )

**Fig. 3-3.** ***Cholesterol Efflux to LpA-I with Varying ApoA-I Number Added to the Medium at the Same Particle Concentration***

Fibroblasts pre-labelled with $^{3}$H-cholesterol (20 μCi/ml) were incubated with medium containing 22.5, 33.75 or 45.0 μg protein/ml of Lp2A-I, Lp3A-I or Lp4A-I respectively under the same conditions as for Fig. 3-2. Medium aliquots were taken at 30, 60 and 90 min of incubation for radioactivity counting. Efflux is expressed as medium cpm/μg cell protein. Each data point presented is the mean of 4 replicate samples and the error bar is the standard deviation.

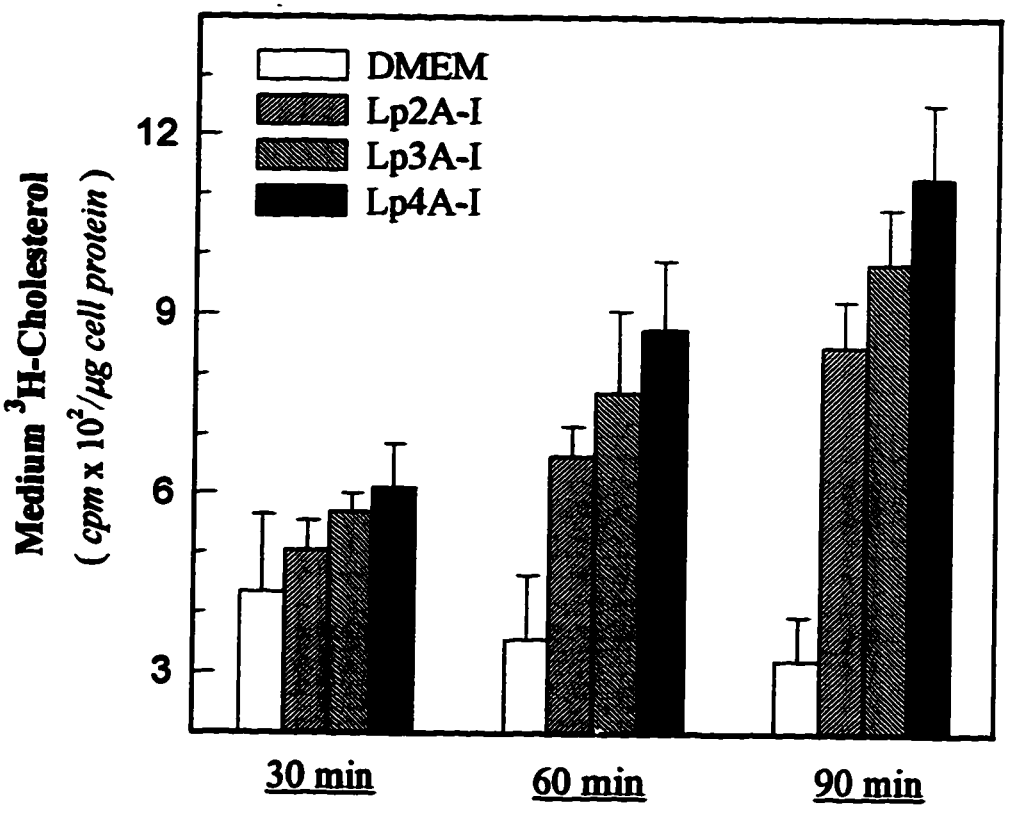

DMEM
Lp2A-I
Lp3A-I
Lp4A-I
Medium $^3$H-Cholesterol
( cpm x $10^2$/μg cell protein )
12
9
6
3
30 min
60 min
90 min

**Fig. 3-4.** ***Effect of Lipoprotein Concentration on Cholesterol Efflux to Lp2A-I and Lp4A-I***

Fibroblasts pre-labelled with $^{3}H$-cholesterol (20 μCi/ml) were incubated in medium containing either Lp2A-I or Lp4A-I at concentrations of 15, 30, 60, 100, 150 and 200 μg protein/ml respectively. Media samples were taken out after 90 min of incubation for radioactivity counting. Panel A represents the cholesterol efflux (radioactivity of medium) plotted as a function of the protein concentrations of these different LpA-I particles. Panel B represents the cholesterol efflux plotted as a function of the different particle concentration in the medium. Each data point presented is the mean of 4 replicate samples and the error bar is the standard deviation.

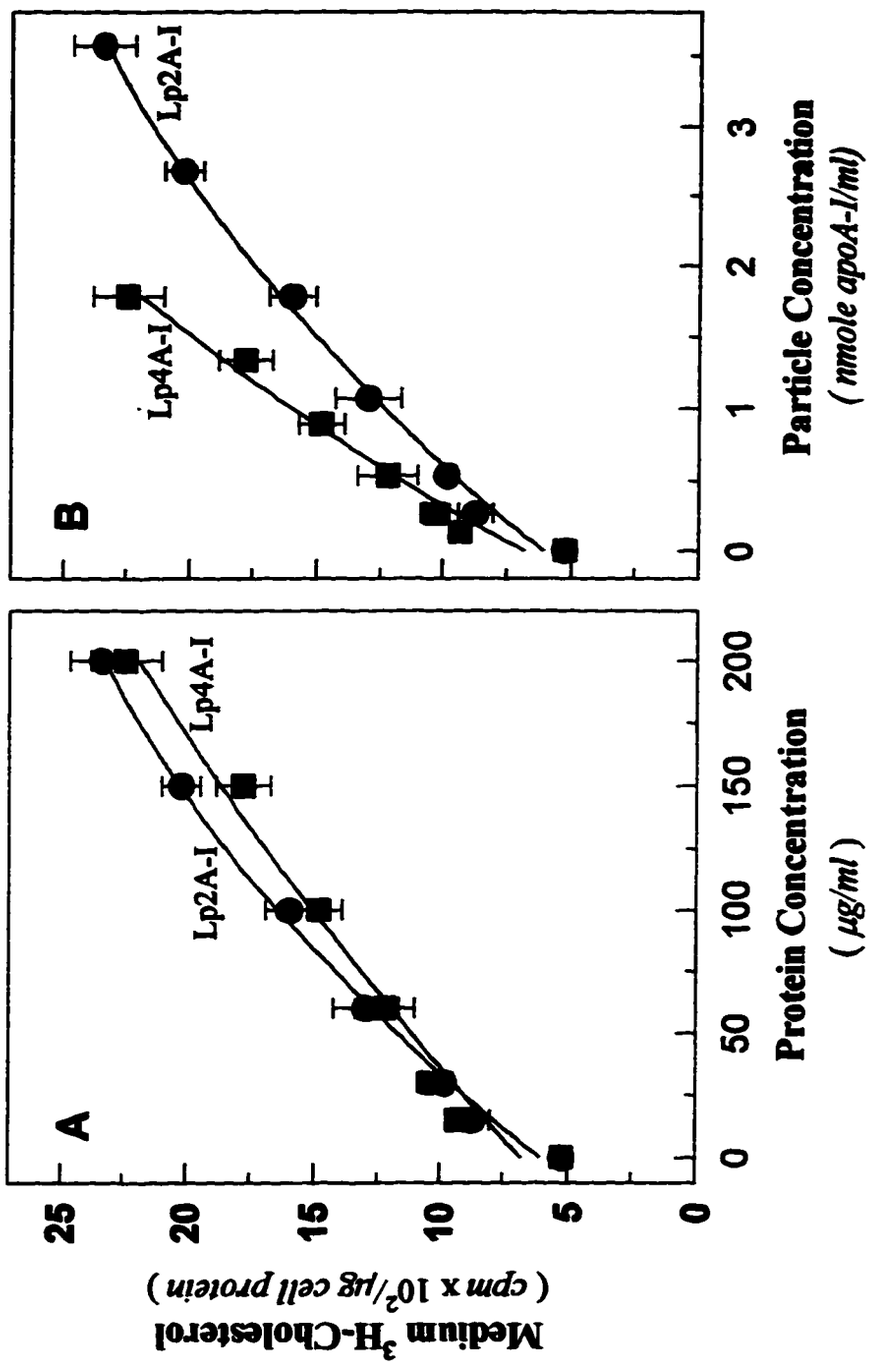

A
B
Lp2A-I
Lp4A-I
Lp2A-I
Lp4A-I
Medium $^3$H-Cholesterol
( cpm x $10^2$/µg cell protein )
Protein Concentration
( µg/ml )
Particle Concentration
( nmole apoA-I/ml )
0
5
10
15
20
25
0
50
100
150
200
0
1
2
3

**Fig. 3-5.** ***Cholesterol Efflux to Lp2A-I with Varying POPC Ratios***

Fibroblasts pre-labelled with $^{3}$H-cholesterol (20 μCi/ml ) were incubated with discoidal Lp2A-I particles (45 μg protein/ml ) with varying POPC/apoA-I molar ratio of 61/1, 100/1 or 133/1, identified as D1, D2 and D3 respectively (see Table 3-I for details of their composition). Aliquots of medium were taken between 2 and 90 min of incubation for the measurement of efflux as described for Fig. 3-2 (n=4, +/- SD).

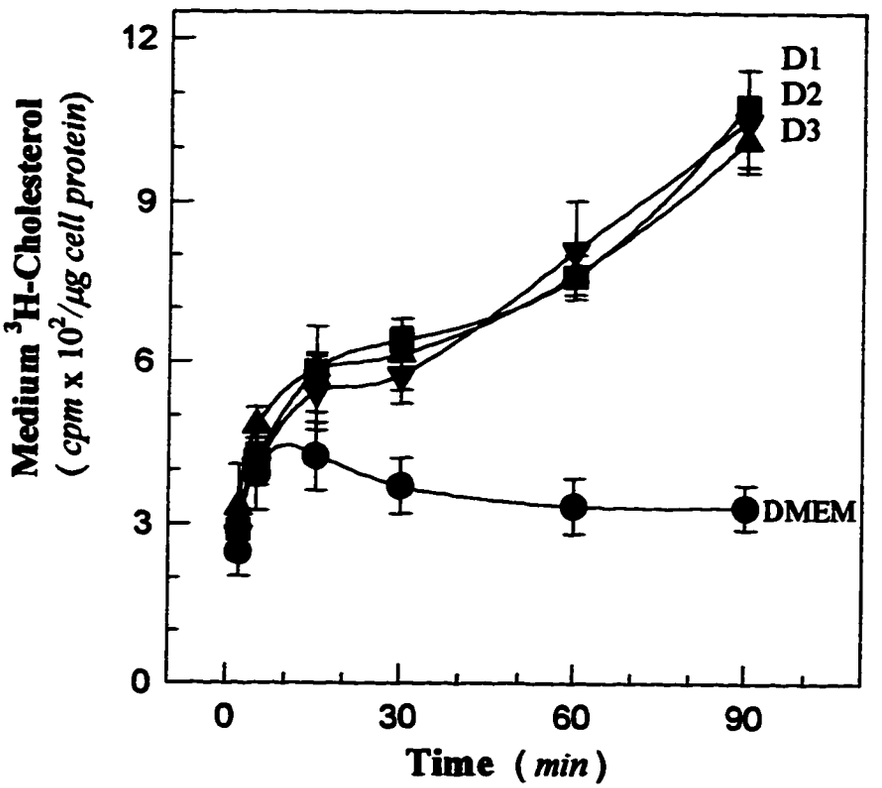

Medium $^3$H-Cholesterol
( cpm x $10^2$/μg cell protein)
12
9
6
3
0
0
30
60
90
Time ( min )
D1
D2
D3
DMEM

**Fig. 3-6.** ***Cholesterol Efflux to Lp2A-I with Varying Ratios of Free Cholesterol***

Fibroblasts pre-labelled with $^{3}$H-cholesterol (20 μCi/ml ) were incubated with discoidal Lp2A-I particles (45 μg protein/ml ) containing 3.4 or 7.7 moles of free cholesterol identified as C2 and C3 respectively. Aliquots were taken between 2 and 90 min of incubation and efflux was measured as described for Fig. 3-2, and compared with the efflux to a Lp2A-I particle having similar POPC/apoA-I ratio but no free cholesterol (C1), (n=4, +/- SD).

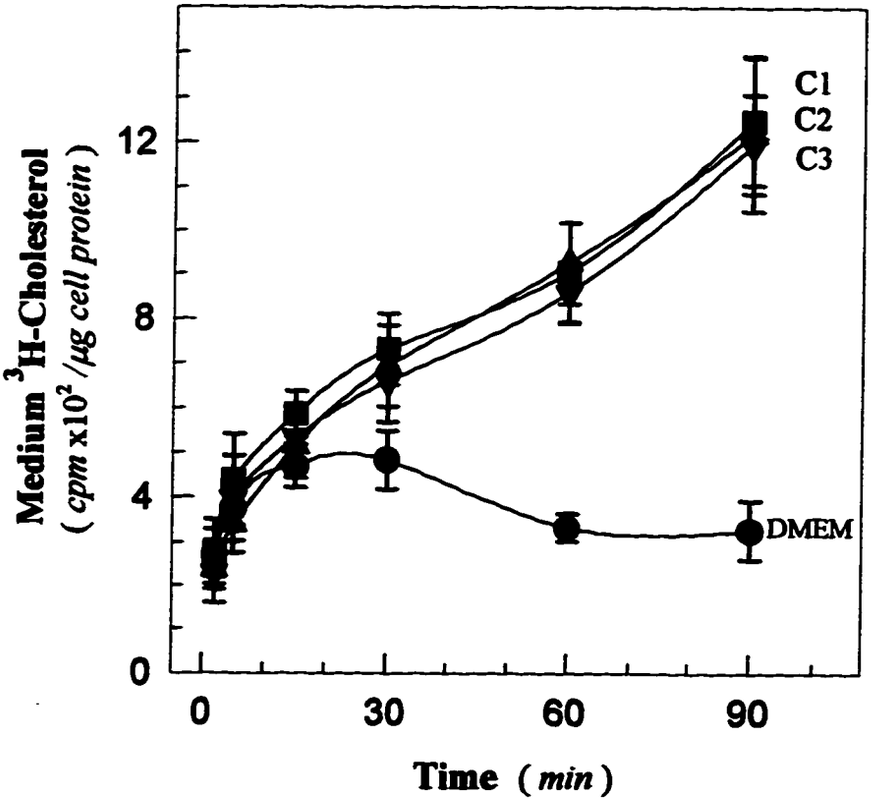

Medium $^3$H-Cholesterol
( cpm x10$^2$ /μg cell protein )
12
8
4
0
0
30
60
90
Time ( min )
C1
C2
C3
DMEM

**Fig. 3-7.** ***Cholesterol Efflux to Lp2A-I with Varying Levels of Phosphatidylinositol***

Fibroblasts pre-labelled with $^{3}$H-cholesterol (20 μCi/ml ) were incubated with discoidal Lp2A-I particles (45 μg protein/ml ) containing 2.8 or 5.6 moles of phosphatidylinositol identified as PI1, and PI2 respectively. Aliquots were taken between 2 and 90 min of incubation and efflux was measured as described for Fig. 3-2, and compared with the efflux to a Lp2A-I particle having similar composition but no PI (C2), (n=4, +/- SD).

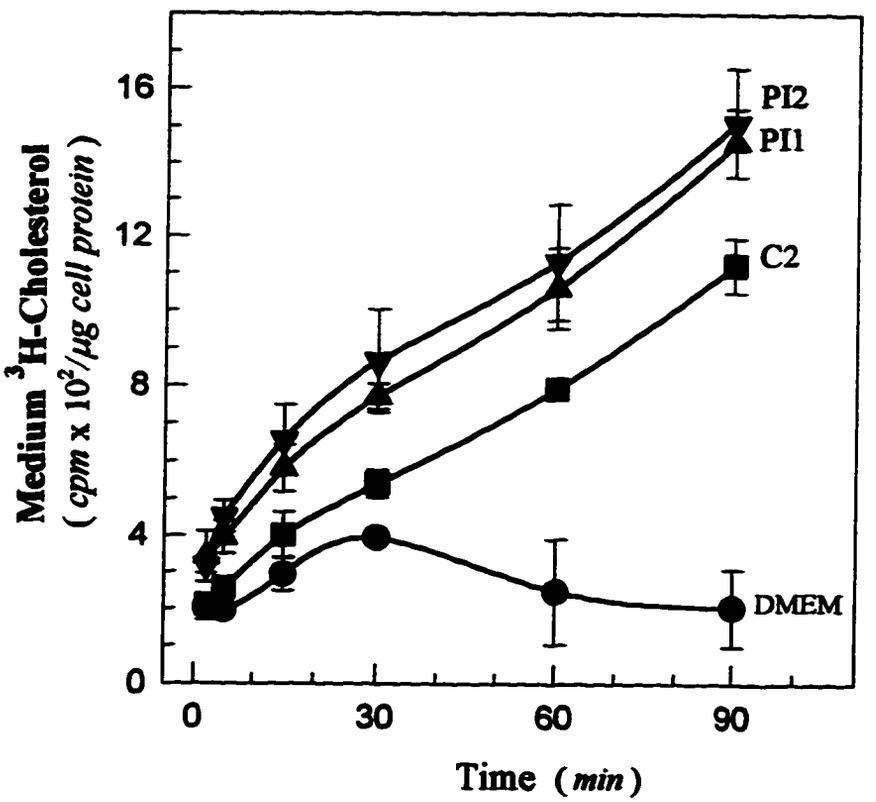

Medium $^3$H-Cholesterol
( cpm x $10^2$/μg cell protein )
16
12
8
4
0
0
30
60
90
Time ( min )
PI2
PI1
C2
DMEM

**Fig. 3-8.** ***Cholesterol Efflux to Lp2A-I with Varying Levels of Sphingomyelin***

Fibroblasts pre-labelled with $^{3}$H-cholesterol (20 μCi/ml ) were incubated with discoidal Lp2A-I particles (45 μg protein/ml ) containing 4.4, 8.2, or 15.1 moles of SM and identified as SM1, SM2 and SM3 respectively. Aliquots were taken between 2 and 90 min of incubation and efflux was measured as described for Fig. 3-2, and compared with the efflux to a Lp2A-I particle having similar composition but no SM (C2), (n=4, +/- SD).

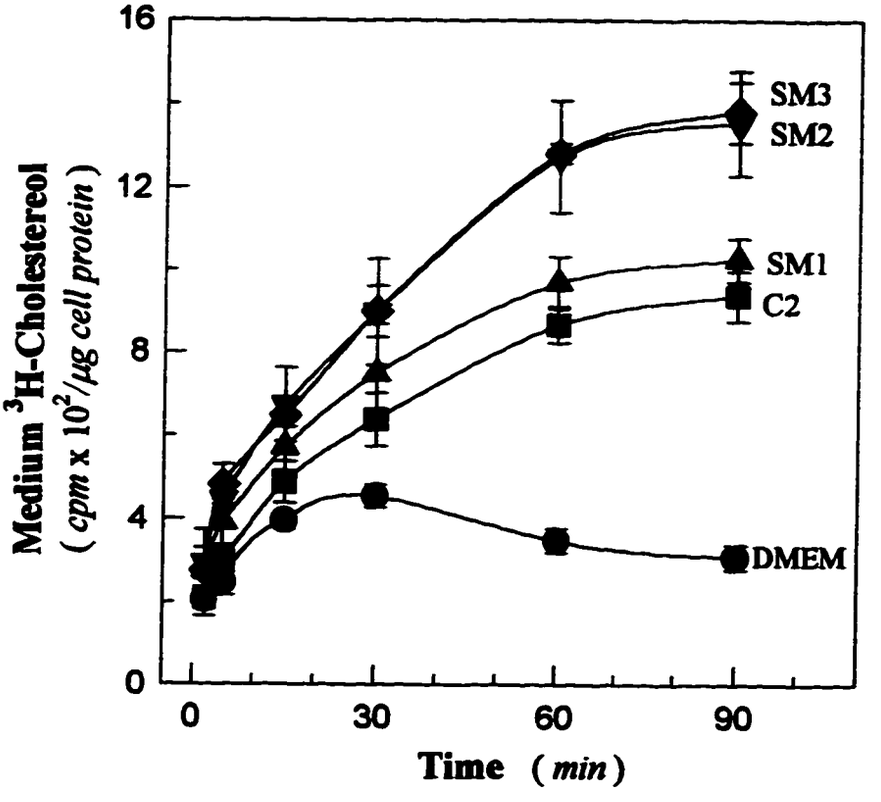
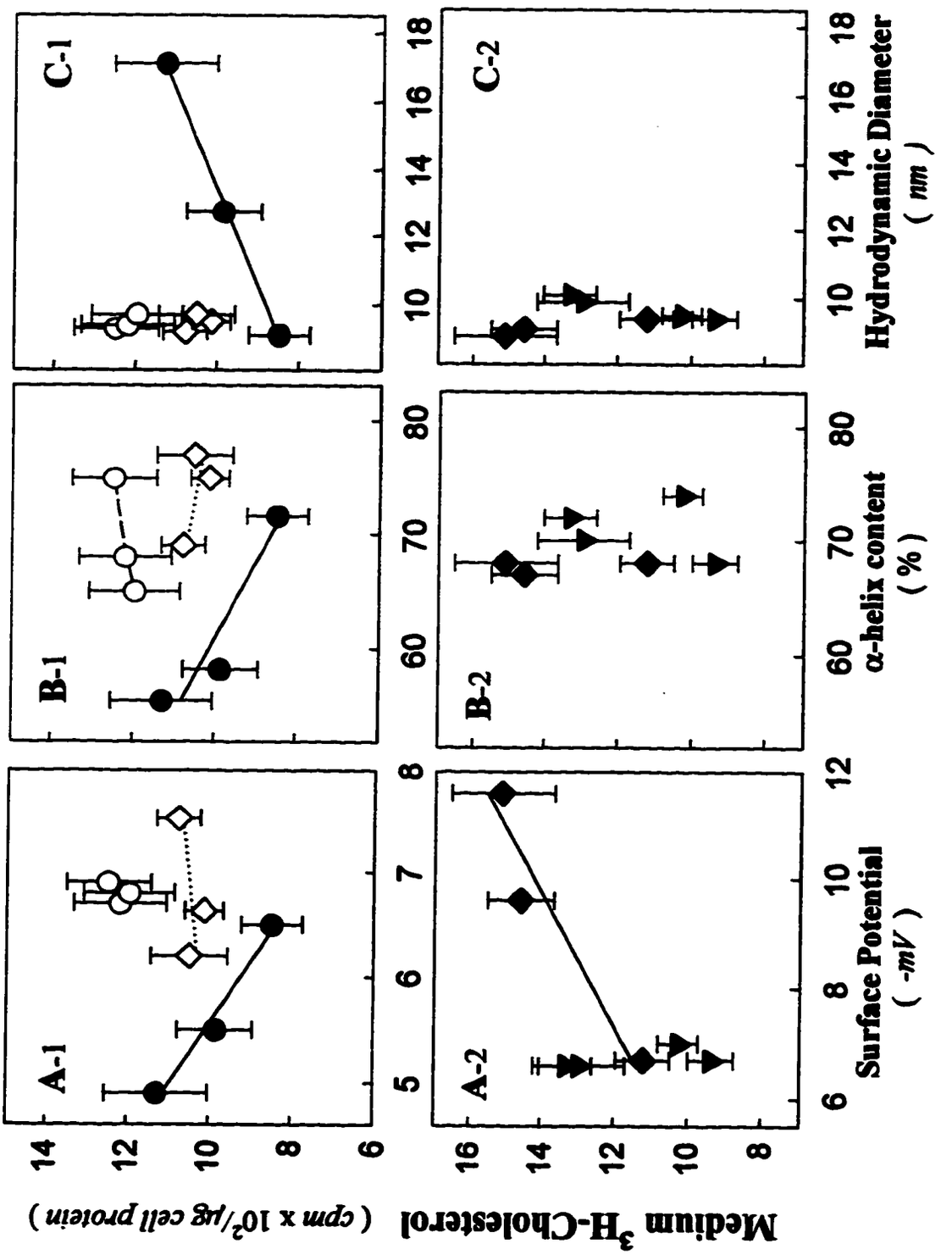

16
12
8
4
0
0
30
60
90
Time (min)
Medium ³H-Cholestereol
(cpm x 10²/μg cell protein)
SM3
SM2
SM1
C2
DMEM

**Fig. 3-9.** ***Relationships Between Discoidal LpA-I Physical Parameters and Their Abilities to Promote Cellular Cholesterol Efflux***

In panels A-1, B-1 and C-1, the filled circles represent the particles containing 2, 3 or 4 apoA-I per particle, the unfilled diamonds represent the Lp2A-I with varying levels of POPC, and the unfilled circles are the Lp2A-I with varying UC contents. In panels A-2, B-2, C-2, the filled diamonds are Lp2A-I with varying PI, and the filled triangles are Lp2A-I with varying SM. For each group of particles, the different panels summarize the relationships between cellular cholesterol efflux at 90 min of incubation and surface potentials (Panel A, A-1), and $\alpha$-helix content (Panel B, B-1), and hydrated diameters (Panel C, C-1).


A-1
B-1
C-1
A-2
B-2
C-2
Medium $^3$H-Cholesterol ( cpm x $10^2$/μg cell protein )
Surface Potential ( -mV )
α-helix content ( % )
Hydrodynamic Diameter ( nm )

# CHAPTER 4

## Specific Phospholipid Association with Apolipoprotein A-I Stimulates Cholesterol Efflux from Human Fibroblasts: Studies with Reconstituted Sonicated Lipoproteins

## I. SUMMARY

To understand how the lipid composition of HDL mediates the efflux of cellular cholesterol, we have characterized the effects of variations in the lipid composition of well-defined reconstituted LpA-I particles on cholesterol efflux from cultured human skin fibroblasts. LpA-I with variations in their content of phosphatidylcholine (POPC), phosphatidylinositol, sphingomyelin, cholesterol ester and triolein were prepared by co-sonication. Association of as little as 5 moles of phosphatidylcholine with apoA-I is sufficient to transform lipid-free apoA-I into a distinct lipoprotein-like particle that is a significantly better acceptor of cellular cholesterol than lipid-free apoA-I. Increasing the ratio of POPC/apoA-I from 5/1 to 35.5/1 in the sonicated LpA-I is associated with a significant increase in the release of cellular cholesterol. At low POPC/apoA-I ratios, native gradient gel electrophoresis of the LpA-I show these lipoproteins to be small complexes (around 5-6 nm), with only one molecule of apoA-I (Lp1A-I). At a POPC/apoA-I

ratio above 11/1, LpA-I form well-defined complexes that contain 2 molecules of apoA-I (Lp2A-I) and range in size from 7.6 to 7.7 nm. Inclusion of sphingomyelin into an Lp1A-I further stimulates cholesterol efflux significantly. In contrast, inclusion of either sphingomyelin or phosphatidylinositol into a sonicated Lp2A-I has no effect on cholesterol efflux. Incorporation of cholesteryl ester and/or triolein into an Lp2A-I particle is associated with a small reduction in cholesterol efflux to these lipoproteins. Therefore, cholesterol efflux from human fibroblasts is directly proportional to the amount and type of phospholipids in a sonicated LpA-I particle. Changes in the conformation and charge of apoA-I that result from changes in the lipid composition of a sonicated LpA-I particle appear to directly affect the ability of the lipoprotein to bind and retain cholesterol molecules. These data therefore suggest that the adsorption/desorption of cholesterol molecules to/from a sonicated LpA-I complex may be less sensitive to interfacial lipid-lipid interactions, but may depend on a conformation-dependent ability of apoA-I to bind cholesterol.

## II. INTRODUCTION

HDL is a highly heterogeneous class of lipoproteins of various origins formed from the catabolism of triglyceride-rich lipoproteins and the secretion of nascent HDL particles by the liver or intestine (Banerjee and Redman, 1983; Eisenberg, 1984, McCall et al., 1988, 1989, Castel et al., 1991, Thrift et al., 1986). Recent studies have suggested that HDL may also be generated by the stepwise lipidation of apoA-I by acquisition of lipids from other lipoproteins (Hussain et al., 1989) or from extrahepatic cells (Hara and Yokoyama, 1991, 1992, Bielicki et al., 1991, 1992, Forte et al., 1993). Supporting evidence for the extracellular assembly process of HDL includes: i) ApoA-I, the predominant apolipoprotein of HDL appears secreted by the liver and the intestine mainly in the lipid free form; however, only about 3% of this apolipoprotein is present in plasma as lipid-free form (Neary and Gowland, 1987); ii) Lipid-free apoA-I is able to release both phospholipid and cholesterol from extrahepatic cells (Bielicki et al., 1992, Hara and Yokoyama, 1991), and incubation of lipid-free apoA-I with Chinese hamster ovary cells in serum-free medium generates LpA-I with a gradual size increment (Forte et al., 1993). A subspecies of small particles (7.3 nm) composed of 94% apoA-I and 6% phospholipids has also been identified in these studies which appears analogous both in composition and size to the human plasma pre β-HDL reported earlier (Kunitake et al., 1985), and may be analogous to the particles

reported to be highly active in cellular cholesterol efflux (Castro and Fielding, 1988).

When lipid-free apoA-I is incubated with extrahepatic cells, the incorporation of phospholipids into apoA-I does not seem to parallel that of cholesterol as evidenced by the facts that: i) The acquisition of phospholipid and cholesterol by human apoA-I from isolated microsomal membrane (Nunez and Swaney, 1984) as well as from intact human fibroblasts or mouse microphages (Li et al., 1993, Li and Yokoyama, 1995, Beliecki et al., 1992; Yancey et al., 1995) is non-stoichiometric; ii) Phospholipid efflux to lipid-free apoA-I precedes and facilitates the efflux of cholesterol (Yancey et al., 1995); iii) Association of phospholipids with apoHDL greatly increases its ability to release cellular cholesterol compared to the delipidated apoHDL (Stein and Stein, 1973), while treatment of HDL with either phospholipase A2 or heparin-releasable rat hepatic lipase reduced cholesterol efflux (Johnson et al., 1986). Therefore, it appears that the formation of apoA-I/phospholipid complexes at the early stage is an important preliminary step before apoA-I can significantly promote the efflux of cellular cholesterol.

However, there is up to now no direct evidence for a relationship between the progressive lipidation of apoA-I and the abilities of the corresponding complexes to release cellular cholesterol in short term incubations, and the

corresponding changes in some physical parameters assumed by the apoA-I/lipid complexes during their progressive lipidation. We have attempted to answer these questions by preparation of model complexes generated in vitro by co-sonication of phospholipid and apolipoprotein as described previously (Hirz and Scanu, 1970, Sparks et al., 1995a). We have first prepared a series of reconstituted sonicated LpA-I complexes with varying POPC/apoA-I ratios in order to answer the above questions. In addition, two other series of POPC/apoA-I complexes have also been prepared to investigate the contribution of HDL lipid composition, such as surface phospholipid components of sphingomyelin (SM) and phosphatidylinositol (PI), or core neutral lipids of cholesterol ester (CE) and triolein (TG) to cellular cholesterol efflux. These studies aim to prepare reconstituted LpA-I which mimic the high affinity acceptor, pre$\beta_1$-LpA-I, that have been identified in whole plasma or lymph (Fielding and Fielding, 1995), and to investigate the progressive incorporation of different lipid composition into apoA-I on the function in cellular cholesterol efflux.

# III. RESULTS

## 1. *Characterization of LpA-I complexes prepared by co-sonication of POPC and ApoA-I*

Co-sonication of POPC and apoA-I forms well-defined POPC/apoA-I complexes, wherein the POPC content can be changed by varying the POPC/apoA-I ratio in the initial mixture. POPC/apoA-I complexes are separated from the lipid-free apoA-I and from the non-incorporated lipid by size exclusion chromatography (not illustrated). As shown in Fig. 4-1, when the initial molar ratio of POPC/apoA-I is below 20/1, the purified POPC/apoA-I complexes appear as well-defined particles on gradient acrylamide gels with sizes at about 5-6 nm. In contrast, large homogeneous LpA-I can be generated with initial POPC/apoA-I molar ratios at and above 20/1. These sonicated LpA-I particles prepared from the initial molar ratios of 20/1, 30/1 and 60/1 have estimated Stoke's diameters on NDGGE of 7.6, 7.6 and 7.7 nm respectively. Chemical cross-linking of the POPC/apoA-I complexes prepared from an initial POPC/apoA-I ratio below 20/1 indicates the presence of only one mole of apoA-I per complex, while those formed at 20/1 and above contain two moles of apoA-I per particle. The composition of these sonicated POPC/apoA-I complexes re-isolated by gel filtration is summarized in Table 4-I. The co-sonication of POPC and apoA-I allows the production of POPC/apoA-I complexes with as little as 2.4 moles of

POPC per mole of apoA-I. The lipid/protein ratios of these lipid-poor LpA-I complexes are analogous to that of the pre β HDL found in normolipidemic human plasma (Kunitake et al., 1985), and the sizes of the complexes generated at the low ratios of POPC/apoA-I are analogous to that of pre$\beta_1$-LpA-I (Castro and Fielding, 1988, Francone and Fielding, 1990, Fielding and Fielding, 1995a).

The molar range of POPC that can be incorporated with apoA-I and allows the formation of a homogenous population of particles varies from a few moles to a maximum of about 36 moles per mole of apoA-I for sonicated LpA-I complexes, while from 40 to 133 moles of POPC are needed to form a homogeneous discoidal LpA-I as shown in Chapter 3. Although the increases of POPC/apoA-I ratios alter only slightly the sizes of the POPC/apoA-I complexes as estimated on NDGGE, the secondary structure of apoA-I does change with the progressive increase of POPC in the complexes, as demonstrated by the modification in α-helical contents and electrophoretic mobilities. The α-helical content increases slightly with the first addition of 2.4 moles of POPC into apoA-I and continues to increase as more POPC associates with apoA-I (Table 4-I). Upon electrophoresis, POPC/apoA-I complexes prepared by sonication migrate to intermediate positions between pre β and α HDL, which tend to decrease with the increase of POPC/apoA-I ratio (Fig. 4-2A). The change in the surface charge of these complexes follows the same pattern as their electrophoretic mobilities (Table 4-I).

## 2. *Characterization of LpA-I complexes with varying ratios of surface phospholipids and core neutral lipids*

After phosphatidylcholine (PC), SM and PI are the two major phospholipids associated with HDL. SM is also one of the major phospholipids of nascent LpA-I including those described as high affinity acceptors for cellular cholesterol (Castro and Fielding, 1988; Hara and Yokoyama, 1991; Fielding and Fielding, 1995). We have tested a large range of ratios of SM or PI/POPC in order to mimic the different phospholipids to apoA-I molar ratios found in typical spherical LpA-I. We have observed that SM competes effectively with POPC for association with apoA-I. Typically, starting from a molar ratio of 40:1:4:20 (POPC:apoA-I:UC:SM), we obtained purified Lp2A-I particles with ratios of 14:1:2.1:11.2, as shown in Table 4-II. The presence of SM does not significantly change the particle size (Table 4-II) or electrophoretic mobility (Fig. 4-2B). The inclusion of PI in the sonicated particle also does not modify their size (Table 4-II), but as should be expected, PI significantly increases the electrophoretic mobility (Fig. 4-2B). We have also generated an SM containing complex with a low phospholipid/apoA-I ratio (S9, Table 4-II) which is still an Lp1A-I particle with a small size (less than 6 nm) and interestingly an electrophoretic mobility close to the pre β range (Fig. 4-2B).

Four subspecies of sonicated spherical LpA-I with CE and/or TG have been made as indicated in Table 4-II. Three moles of UC, 6 moles of CE and/or 6 moles of TG have been added to POPC/apoA-I mixtures with an initial molar ratio of 60/1, a ratio which we have shown to generate homogenous "spherical" LpA-I particles. Compared to the LpA-I particles containing only POPC and apoA-I, the presence of UC, CE and/or TG does not affect the homogeneity of these sonicated LpA-I particles as shown by NDGGE. The presence of UC reduces slightly the level of POPC that can be incorporated into apoA-I; however, at the molar ratios used here, the presence of TG and/or CE increases POPC incorporation into LpA-I. TG and CE by themselves can be very efficiently incorporated into LpA-I with TG having an incorporation efficiency close to 100%. The presence of neutral lipids, CE or TG, alone or in combination does not change significantly the size or the electrophoretic mobility of the sonicated LpA-I (Table 4-II and Fig. 4-2B).

### 3. *Cellular cholesterol efflux from fibroblasts to LpA-I complexes with varying POPC/apoA-I ratios*

ApoA-I in aqueous solution has been reported to be self-associated at high concentrations but monomeric at concentrations below 1 mg/ml (Vitello and Scanu, 1976). However, there is no report in the literature on the potential impact of apoA-I aggregation on cholesterol efflux to lipid-free apoA-I. To study this possibility, we analyzed cholesterol efflux to apoA-I, either stored at a

concentration of about 1.4 mg/ml and diluted into 45 μg/ml immediately before efflux experiment (oligomeric apoA-I), or diluted to 45 μg/ml overnight before the experiment (monomeric apoA-I). Our results show that rates of cholesterol efflux to these pre-diluted or non-pre-diluted apoA-I are very similar, indicating that conditions favoring apoA-I self-association have no effect on cholesterol efflux. Similarly, we have verified that the sonication procedure used for the preparation of LpA-I complexes does not modify the ability of apoA-I to release cellular cholesterol since similar efflux rates are observed for lipid-free apoA-I subjected or not to the sonication procedure (Fig. 4-3).

In contrast to discoidal LpA-I, where variations in POPC content have been shown to have no effect on the ability of these lipoproteins to accept cholesterol (Chapter 3), increase in the POPC content of sonicated POPC/apoA-I complexes significantly enhance their abilities to promote the efflux of cellular cholesterol (Fig. 4-4). Within the first 90 min of incubation, the efflux also follows the bi-phasic pattern usually observed with fibroblasts. The stimulating effect of the phospholipid content of the LpA-I complexes can also be observed in both phases. The second phase of cellular cholesterol efflux to these sonicated LpA-I complexes is linear, and positively related to the phospholipid levels incorporated into LpA-I. No saturation is observed up to 90 min incubation. Cellular cholesterol efflux is very sensitive to the association of phospholipid with apoA-I, as

demonstrated by the significant increase in efflux observed with as few as 2.4 moles of POPC per mole of apoA-I compared to lipid free apoA-I (data not shown). The efflux of cholesterol from fibroblasts to S2 or S4 is concentration dependent up to 100 μg protein/ml, where both particles nearly reach saturation (Fig. 4-5). Again, the LpA-I complex containing more POPC shows higher ability to accept cell-derived cholesterol at all concentrations tested. The calculated $EC_{50}$ are 28.0 and 26.7 μg/ml respectively for S2 and S4. At all concentrations tested, lipid free apoA-I is not efficient in promoting cholesterol efflux compared to the lipidated apoA-I .

To compare the abilities of discoidal (prepared by cholate dispersion) and sonicated spherical LpA-I to receive cellular cholesterol from cultured human skin fibroblasts, discoidal LpA-I and spherical LpA-I with similar POPC/apoA-I molar ratios (38.7/1 and 35.5/1 respectively) have been prepared from initial POPC/apoA-I molar ratios of 40/1 and 60/1 (Table 4-I). In spite of their similar final POPC and apoA-I composition, these two species of LpA-I differ significantly in their sizes (9.3 versus 7.7 nm), α–helix content, and negative surface potential (Table 4-I). Comparison of cellular cholesterol efflux to these two species LpA-I particles indicated that discoidal LpA-I are more efficient cholesterol acceptors than sonicated LpA-I (Fig. 4-4).

### 4. *Effect of surface phospholipids and core neutral lipid composition of sonicated LpA-I on cellular cholesterol efflux*

The cellular cholesterol efflux induced by sonicated Lp2A-I with surface phospholipids or with core neutral lipids during a 90 min incubation also follows a bi-phasic pattern (Fig. 4-6A, 4-6B, 4-7A, B, C). In contrast to what has been observed with reconstituted discoidal Lp2A-I (Chapter 3), addition of SM to sonicated Lp2A-I particles at the ratios of 14/1/2.1/11.2 (POPC/apoA-I/UC/SM) does not change the rate of cholesterol efflux (Fig. 4-6A). Neither is the addition of PI to these Lp2A-I (Fig. 4-6A). The high affinity acceptors for cellular cholesterol described as pre$\beta_1$ LpA-I (Castro and Fielding, 1988) are small complexes with only one apoA-I and a low ratio of POPC and SM to apoA-I. We prepared similar complexes by co-sonication of POPC and SM with apoA-I (10/10/1, starting molar ratio) and re-isolation by gel filtration. Compared to a control particle containing POPC and apoA-I (20/1, starting molar ratio), the efflux to the SM-containing complex was significantly faster in both phases as shown in Fig. 4-6B.

The rates of cellular cholesterol efflux induced by sonicated LpA-I with either a single neutral core lipid or both TG and CE are slightly decreased compared to sonicated LpA-I with UC only (Fig. 4-7). However, these differences are not statistically significant. In a separate experiment, it has also been observed

that the presence of 2 moles of UC in sonicated LpA-I does not affect cellular cholesterol efflux compared to sonicated LpA-I with POPC and apoA-I only (Fig. 4-8).

# IV. DISCUSSION

Studies have shown that discoidal LpA-I particles are excellent acceptors of cellular cholesterol (Johnson et al., 1986, 1991, Agnani and Marcel, 1993, Jonas et al., 1994) but that these lipoproteins differ from plasma resident native HDL particles, both in their shape and structure (Sparks et al., 1992a, c). Sonicated LpA-I particles have been shown to have a spherical shape and to be similar to native LpA-I by a variety of structural criteria (Sparks et al., 1992a, c, 1995b). Initial rates of cholesterol efflux measured during the first 5 min are similar for discs and spheres but efflux to discoidal LpA-I increases more rapidly over time than for spheres. The increased ability of discoidal LpA-I to act as a cholesterol acceptor may be partially due to a larger lipid interfacial surface area which may allow for a higher capacity to bind cholesterol molecules released from cell plasma membrane (Phillips et al., 1987, Davidson et al., 1995). It is also possible that the higher stability of discoidal LpA-I relative to sonicated LpA-I (Sparks et al., 1992a, 1995a) may affect the ability of these lipoproteins to retain cholesterol molecules.

In the present study, we observed a quantum jump in the ability of apoA-I to bind cellular cholesterol after complexation with POPC. A comparison of rates of cholesterol efflux to lipid-free apoA-I relative to the sonicated complex S2 shows that addition of only 5 moles of POPC to apoA-I is sufficient to nearly

double the initial rate of efflux (Fig. 4-4). Lipid-poor apoA-I, as well as apoA-II, apoA-IV and apoE have been identified both in plasma (Neary and Gowland, 1987, Ishida et al., 1987) and in the interstitial fluid/peripheral lymph (Sloop et al., 1983a, b, 1987; Reichl et al., 1989). The formation of small HDL by the interaction of lipid-free apolipoproteins with cell plasma membrane lipids may play an important role in the efflux of cholesterol from extrahepatic cells (Bielecki et al., 1991, 1992, Hara and Yokoyama, 1991, 1992). The non-synchronized efflux of cholesterol and phospholipid induced by lipid-free apoA-I (Nunez and Swaney, 1984, Forte et al., 1993, Bielecki et al., 1992, Yancey et al., 1995) and the data presented here are consistent with the hypothesis that the amount and type of phospholipid incorporated into apoA-I will be a controlling factor for the efflux of cellular cholesterol. The Lp1A-I complexes generated by sonication at low phospholipid/apoA-I ratio exhibit a composition that appears to be analogous to that of pre $\beta_1$-LpA-I identified by Fielding and colleagues (Castro and Fielding, 1988, Miida et al., 1990, 1992, Francone et al., 1989, 1990, Fielding and Fielding, 1995). The apparent Stoke's diameters of the sonicated Lp1A-I also compare well with that previously reported for pre $\beta_1$-LpA-I (Asztalos et al., 1993, Fielding and Fielding, 1995). Sonication can therefore generate complexes, which at low POPC/apoA-I ratio, mimic both the structural and functional properties of pre $\beta_1$ LpA-I.

Our data show that the ability to release cellular cholesterol is directly related to the amount of POPC associated with apoA-I, and that this effect can be observed with as little as 5 molecules of POPC per molecule of apoA-I. This is completely in contrast to what has been observed with discoidal LpA-I, where variations in POPC content had no effect on the ability of these lipoproteins to retain cholesterol molecules (Chapter 3). This unique effect of phospholipid on efflux to sonicated LpA-I may simply reflect a requirement for a substantial lipid milleu to solubilize cholesterol molecules. Alternately, this effect of phospholipids may reflect changes in the collisional properties of the lipoprotein resulting from changes in the electrostatic properties of the LpA-I particles. The increased ability of apoA-I to retain cellular cholesterol as the phospholipid/apoA-I ratio is increased in sonicated LpA-I is inversely correlated with the negative surface potentials of these complexes (Fig. 4-9). This may suggest that reducing the net negative charge on an HDL particle may increase the collisional frequency and the rate of cholesterol transfer from cell plasma membrane. Changes in the phospholipid/apoA-I ratios of the sonicated LpA-I may also affect the ability of cholesterol to bind directly to apoA-I. Previous reports have shown that the apoA-I conformation changes substantially upon association with phospholipids (Jonas et al., 1989, Sparks et al., 1992a, c, 1993). Epitope expression investigations in this laboratory have further shown that incorporation of few phospholipid molecules in

sonicated LpA-I directly affects the epitope exposure in a central region of apoA-I (Marcel et al., unreported results), a region that has been proposed to be a cholesterol binding domain (Sparks et al., 1993, Bergeron et al., 1995). The increased ability of apoA-I to retain cellular cholesterol upon association with phospholipids is also significantly correlated with the α-helix contents of these complexes (Fig. 4-9). This differential effect of phospholipids on cholesterol efflux to spheres and discs may be related to alterations in the apoA-I α-helix thermodynamic stabilities on the different complexes. On sonicated LpA-I particles, apoA-I exhibits a thermodymanic stability that is less than that observed for lipid-free apoA-I ($\Delta G_D^o$<2.5 kcal/mol apoA-I, Sparks et al., 1995). On these lipoproteins, apoA-I would thermodynamically prefer to be in a lipid-free state. Addition of small amounts of cholesterol to these particles increases the stability of apoA-I (Sparks et al., 1993) and therefore cholesterol retention in these particles would be thermodynamically favored. In contrast, discoidal LpA-I are generally more stable ($\Delta G_D^o$>2.5 kcal/mol apoA-I) than lipid-free apoA-I. This may render these lipoproteins less metastable and minimize the variability in the propensity of discoidal LpA-I to retain cholesterol, while at the same time may allow for a relative high capacity for lipid storage.

Our interest in testing the effect of SM on the ability of LpA-I complexes to sustain cholesterol efflux stems from several different observations. There has

been a number of studies that have shown that cholesterol has a greater affinity for SM than for PC (Fugler et al., 1985, Yeagle and Young, 1986, Lund-Katz et al., 1988,). Pre$\beta_1$ LpA-I complexes have been reported to be the most avid cholesterol acceptor and have also been shown to contain a high level of SM (Fielding and Fielding, 1995). Finally, a positive relation between the SM contents of a series of sonicated SM-liposomes and their abilities to release cellular cholesterol from human skin fibroblasts has been reported (Stein et al., 1988). At low phospholipid/apoA-I ratios in Lp1A-I, we observed a significant increase in initial rates of efflux when about half of POPC was replaced with SM (Fig. 4-6B). This is consistant with the hypothesis that small pre $\beta_1$-LpA-I complexes, which contain equimolar amounts of both PC and SM, are avid and efficient cholesterol acceptors (Fielding and Fielding, 1995a). It is interesting to note that cholesterol efflux to these complexes is increased even at the earliest two minute time point. This may support the hypothesis proposed by Fielding and Fielding (1995a), that cholesterol transferred to pre $\beta_1$-LpA-I is retained because SM reduces its off-rate as it does in synthetic membranes (Kan et al., 1991). At high ratios of POPC/apoA-I in Lp2A-I, however, the inclusion of SM or PI does not have any effect on cholesterol efflux. This is in contrast to what has been observed with discoidal LpA-I, where inclusion of SM and PI both stimulated cholesterol efflux into the LpA-I particles. Taken together these data suggest that the effect of SM on

cholesterol efflux is not likely to be due to be a purely lipid affinity effect, but may instead result from unique changes in apoA-I conformation and stability. Studies have shown that changes in the lipid composition of discoidal and spherical LpA-I have unique effects on the conformation of apoA-I (Collet et al., 1991, Sparks et al., 1992a, b, Bergeron et al., 1995). Since changes in apoA-I conformation may also affect the ability of cholesterol to bind to this apolipoprotein (Sparks et al., 1993), it seems possible that differences in the effect of SM and PI on cholesterol efflux to spheres and discs may reflect apoA-I conformational differences that directly modify the interactions between cholesterol and apoA-I.

# V. CONCLUSION

We have shown that cholesterol efflux from human skin fibroblasts is directly proportional to the amount and type of phospholipids in a sonicated LpA-I particle. The incorporation of as little as 5 moles of POPC is critical to turn lipid-free apoA-I into an avid recipient of cell-derived cholesterol, and the presence of SM in Lp1A-I further stimulates the efflux. In contrast to the investigations with discoidal LpA-I, cholesterol efflux to sonicated LpA-I appears to be affected by specific changes in apoA-I conformation and surface charge. Changes in the conformation and charge of apoA-I that result from changes in the lipid composition of a sonicated LpA-I particle appear to directly affect the ability of the lipoprotein to bind and retain cholesterol molecules.

a. Results are the everages of at least three preparations.

b. The POPC/apoA-I molar ratios of the initial mixtures for the preparation of LpA-I.

c. The POPC/apoA-I molar ratios of reconsituted LpA-I after re-isolation. The intersample variation is <5%.

d. Estimated by protein cross-linking with DMS and subsquent SDS/PAGE.

e. Total phospholipid content of each reconstituted LpA-I particle.

f. Hydrodynamic diameters of LpA-I determined from NDGGE (±0.5 nm SD). Particles for which size is not given are either heterogenous or had a size outside of the standard range.

g. Determined from molar ellipticities at 222 nm in spectra (±4% SD).

h. Calculated from the electrophoretic migration of LpA-I on agarose gel (±0.2 mV SD).

## TABLE 4-I

*Characterization of Reconstituted LpA-I with Varying Ratios of POPC/ApoA-I*[a]

| | POPC/apoA-I Molar Ratio | | ApoA-I[d] | POPC[e] | Size[f] | α-Helix[g] | Surf. Poten[h] |
|---|---|---|---|---|---|---|---|
| LpA-I | (Initial)[b] | (Final)[c] | (mol/LpA-I) | | ( nm ) | (%) | (- mV ) |
| apoA-I | apoA-I | 0 : 1 | -- | -- | -- | 47 | 8.3 |
| S1 | 5 : 1 | 2.4 : 1 | 1 | 2.4 | -- | 48 | 10.0 |
| S2 | 10 : 1 | 5.0 : 1 | 1 | 5.0 | -- | 49 | 10.0 |
| S3 | 20 : 1 | 11.1 : 1 | 2 | 22.2 | 7.6 | 51 | 9.6 |
| S4 | 30 : 1 | 16.2 : 1 | 2 | 32.4 | 7.6 | 53 | 9.4 |
| S5 | 60 : 1 | 35.5 : 1 | 2 | 71.0 | 7.7 | 59 | 9.2 |
| D1 | 40 : 1 | 38.7 : 1 | 2 | 79.0 | 9.3 | 55 | 7.8 |

## TABLE 4-II

*Characterization of Sonicated LpA-I Complexes with Sphingomyelin, Phosphatidylinositol or HDL Core Neutral Lipids*[a]

| LpA-I | Composition Molar Ratio (initial)[b] | Composition Molar Ratio (final)[c] | ApoA-I[d] (mol/LpA-I) | Size[f] ( nm ) | Surf. Potent[h] (- mV ) |
|---|---|---|---|---|---|
| **Variation in sphingomyelin and phosphatidylinositol (POPC/ApoA-I/UC/SM/PI)** | | | | | |
| S6 | 60 : 1 : 4 : 0 : 0 | 29 : 1 : 1.8 : 0 : 0 | 2 | 7.7 | 9.6 |
| S7 | 40 : 1 : 4 : 20 : 0 | 14 : 1 : 2.1 : 11.2 : 0 | 2 | 7.8 | 9.1 |
| S8 | 50 : 1 : 4 : 0 : 10 | 24 : 1 : 1.5 : 0 : 6.1 | 2 | 7.5 | 12.9 |
| S9 | 10 : 1 : 0 : 10 : 0 | 4.7 : 1 : 0 : 5.4 : 0 | 1 | < 6 | 8.3 |
| **Variation in neutral core lipids (POPC/ApoA-I/UC/CE/TG)** | | | | | |
| S10 | 60 : 1 : 3 : 6 : 0 | 46 : 1: 1.2 : 2.9 : 0 | 2 | 7.8 | 9.3 |
| S11 | 60 : 1 : 3 : 0: 6 | 38 : 1 : 1.3 : 0 : 6.4 | 2 | 7.7 | 10.1 |
| S12 | 60 : 1 : 3 : 6 : 6 | 43 : 1 : 1.2 : 2.6 : 6.5 | 2 | 7.7 | 10.2 |

a. Legends are the same as in Table I.

**Fig. 4-1.** ***Non-denaturing Gradient Gel Electrophoresis of Sonicated POPC/ ApoA-I Complexes with Progressive Increase of POPC Content***

POPC/apoA-I complexes were prepared by sonication from mixtures of POPC/apoA-I at molar ratios of 2.4:1, 5.0:1, 11.1:1, 16.2:1, 35.5:1, and named as S1, S2, S3, S4, S5 respectively. D1 is a species of discoidal Lp2A-I made from a POPC/apoA-I mixture of 40:1 (molar ratio). After purification by gel filtration, the complexes were electrophoresed on precast NDGGE (8-25%) and stained with Coomassie blue. The lane labelled as STD is the high molecular weight protein standard of thyroglobulin, apoferritin, catalase, lactate dehydrogenase and albumin (n=4, +/- SD).

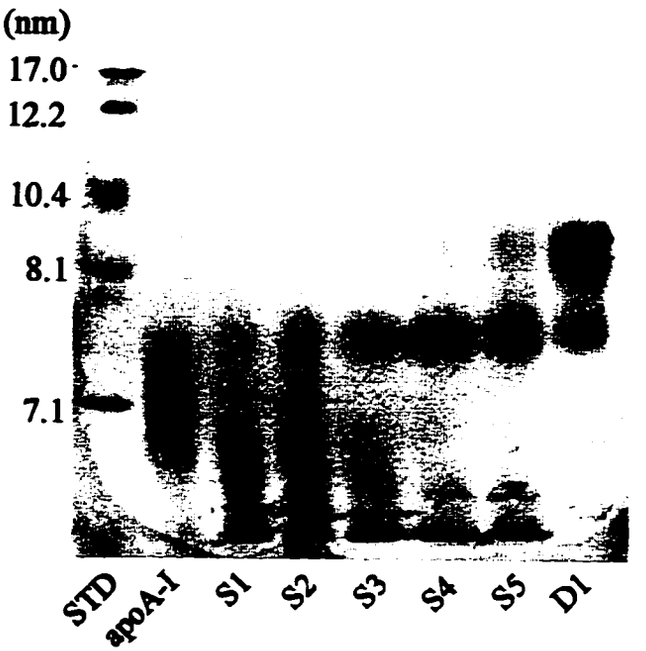

(nm)
17.0
12.2
10.4
8.1
7.1
STD
apoA-I
S1
S2
S3
S4
S5
D1

**Fig. 4-2.** ***Electrophoretic Mobilities of POPC/apoA-I Complexes with or without Sphingomyelin, Phosphatidylinositol and Core Neutral Lipids***

Re-isolated POPC/apoA-I complexes with a progressive increase in POPC content (panel A), and LpA-I complexes with SM, PI, or core neutral lipids (panel B) corresponding to those described in Table 4-I and 4-II were electrophoresed on 0.6% agarose gels for 30 min. The electrophoretic mobilities were calculated from the migration distance of the particles as previously described (Sparks et al., 1992). The standard pre β and α-migration positions were calculated from the migration distance of the corresponding lipoproteins isolated from fresh plasma and electrophoresed under the same conditions. The data are the average of at least four determinations (n=4, +/- SD).

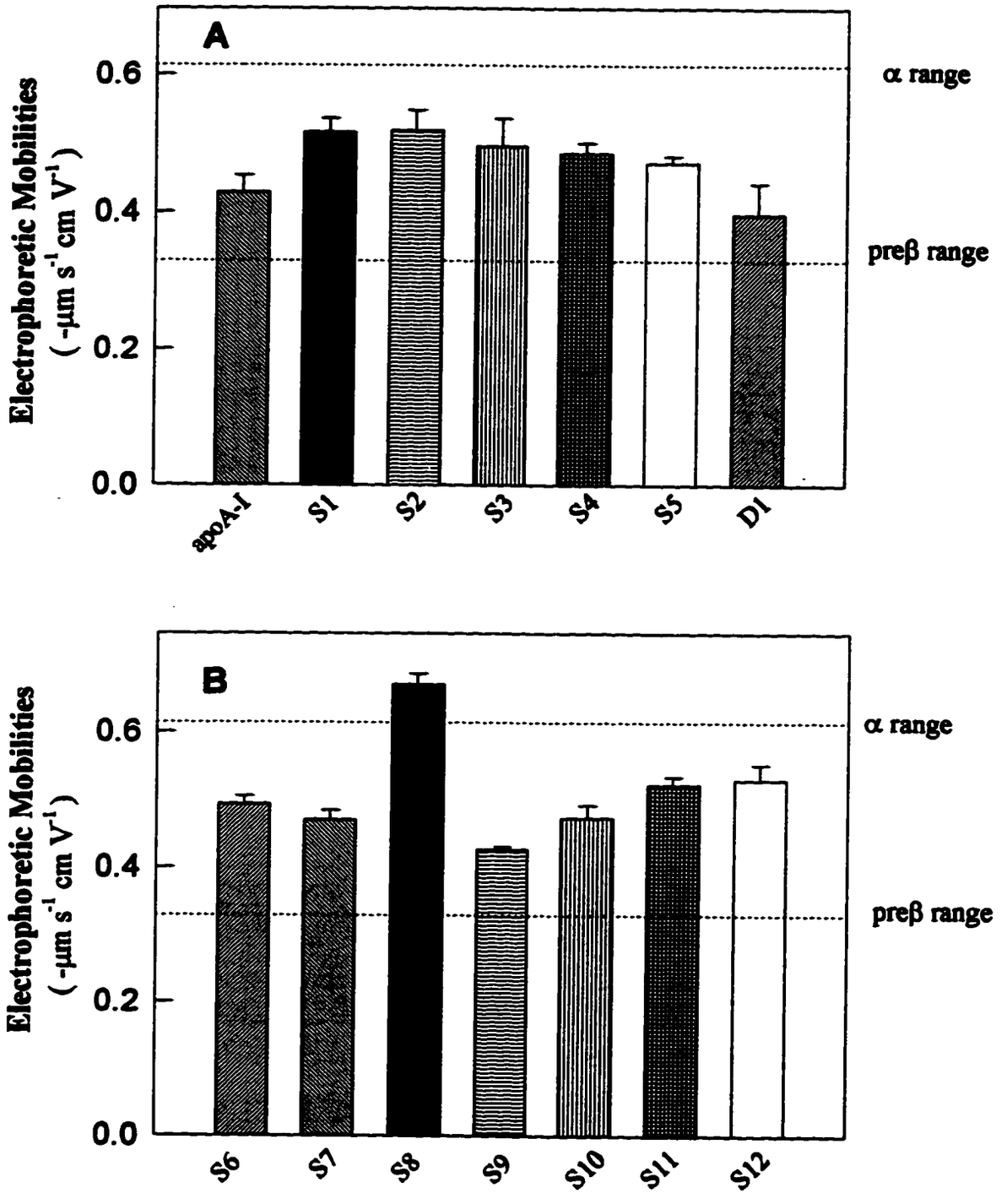

A
Electrophoretic Mobilities
(-μm s-1 cm V-1)
0.6
0.4
0.2
0.0
α range
preβ range
apoA-I
S1
S2
S3
S4
S5
D1
B
Electrophoretic Mobilities
(-μm s-1 cm V-1)
0.6
0.4
0.2
0.0
α range
preβ range
S6
S7
S8
S9
S10
S11
S12

**Fig. 4-3.** ***Cholesterol Efflux to Lipid-free ApoA-I with Different Treatments.***

Human skin fibroblasts at 70% confluence were labelled with 1,2n-$^{3}$H-cholesterol (20 μCi/ml) in the presence of 5% FBS and other supplements for 48 h. For the efflux study, the cells were washed twice with DMEM containing 0.2% essentially fatty acid-free BSA and then twice with DMEM alone. The cells were then incubated with DMEM containing 45 μg/ml of lipid-free apoA-I pre-treated differently as indicated in the figure. DMEM without free apoA-I was used as control. At time intervals between 2 and 90 min, aliquots of medium were taken for radioactivity determination. Cholesterol efflux is expressed as medium radioactivity per μg cell protein (n=4, +/- SD).

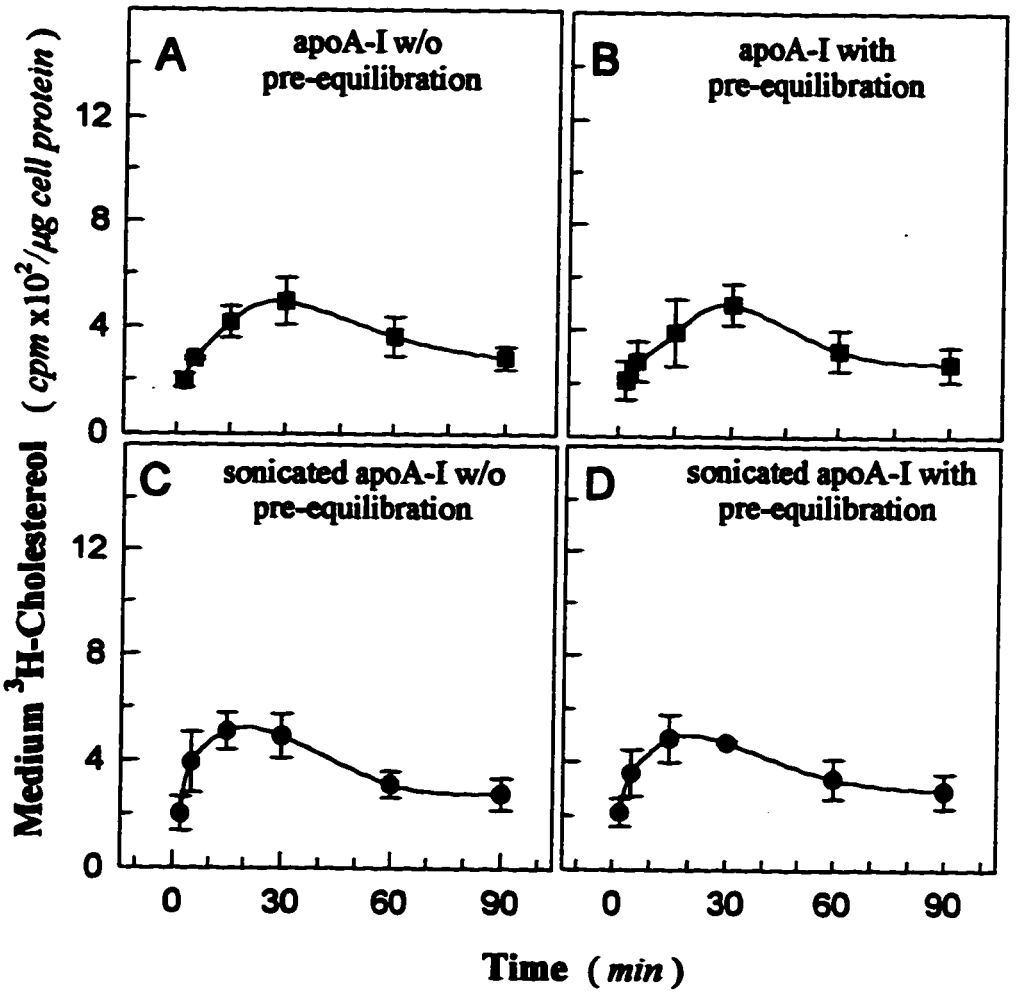

A
apoA-I w/o pre-equilibration
B
apoA-I with pre-equilibration
C
sonicated apoA-I w/o pre-equilibration
D
sonicated apoA-I with pre-equilibration
Medium $^3$H-Cholestereol ( cpm x10$^2$/μg cell protein )
0
4
8
12
0
30
60
90
Time ( min )

**Fig. 4-4.** ***Effect of POPC Content on the Time-dependent Cellular Cholesterol Efflux to POPC/ApoA-I Complexes***

Human skin fibroblasts at 70% confluence were labelled with 1,2n-$^{3}$H-cholesterol, and washed as described in Fig. 4-3. For the efflux study, the cells were incubated with DMEM containing 45 μg protein/ml of sonicated lipid-free apoA-I, or POPC/ apoA-I complexes at a final POPC/apoA-I molar ratio of 5/1, 16.2/1, or 35.5/1 (S2, S4, S5, respectively), or discoidal Lp2A-I (D1) (see Table 4-I for details of their characterization). DMEM containing no cholesterol acceptor was used as control. At the indicated time intervals, aliquots of medium were taken for radioactivity determination. Cholesterol efflux is expressed as medium radioactivity per μg cell protein (n=4, +/- SD).

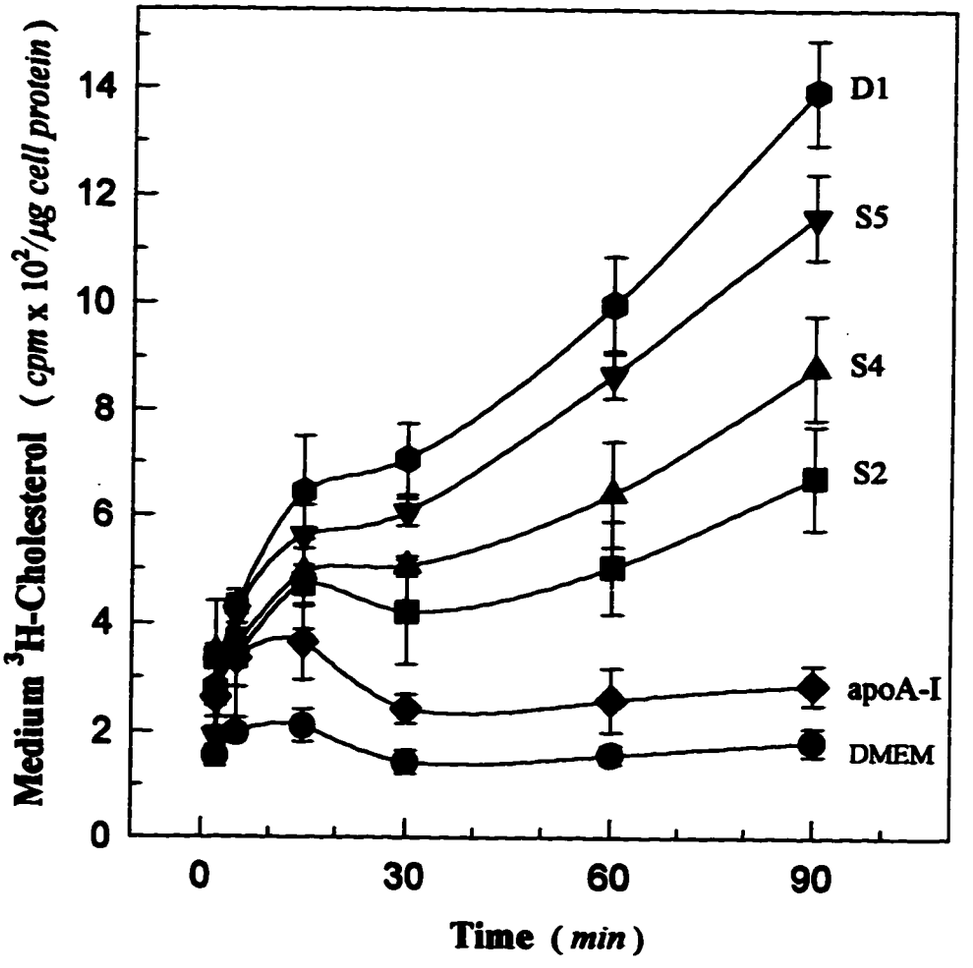

Medium $^3$H-Cholesterol ( cpm x $10^2$/μg cell protein )
0
2
4
6
8
10
12
14
0
30
60
90
Time ( min )
D1
S5
S4
S2
apoA-I
DMEM

**Fig. 4-5.** ***Effect of POPC Content on Concentration-dependent Cellular Cholesterol Efflux to POPC/ApoA-I Complexes***

Human skin fibroblasts were seeded and labelled as described in Fig. 4-3. For the efflux study, the washed cells were incubated with DMEM containing sonicated lipid-free apoA-I, or POPC/apoA-I complexes at the final POPC/apoA-I molar ratio of 5/1 or 16.2/1 (S2 and S4 respectively). Media aliquots were taken at 90 min incubation for radioactivity determination. Efflux is expressed as medium radioactivity per μg cell protein (n=4, +/- SD).

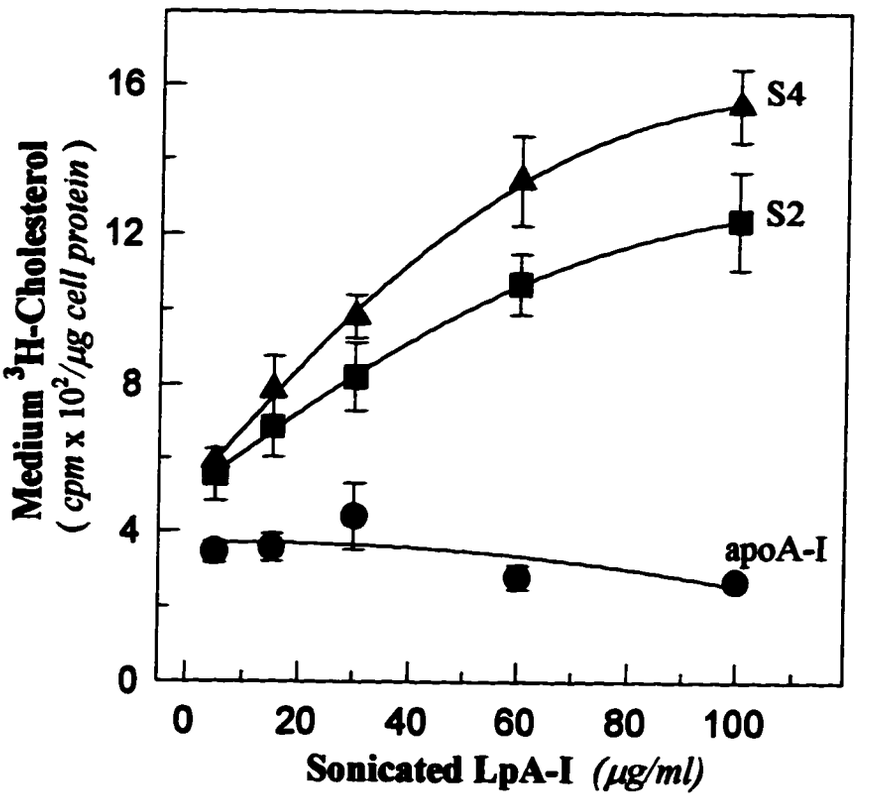
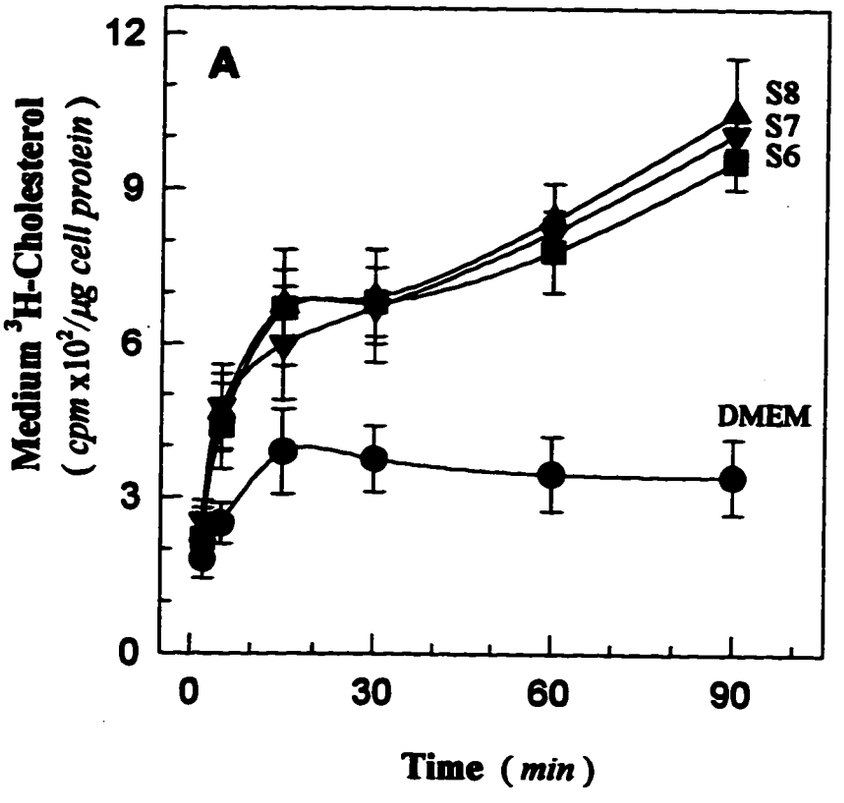

Medium $^3$H-Cholesterol
( cpm x $10^2$/μg cell protein )
16
12
8
4
0
0
20
40
60
80
100
Sonicated LpA-I (μg/ml)
S4
S2
apoA-I

**Fig.4-6.** ***Effect of Sphingomyelin or Phosphatidylinositol Content on Cholesterol Efflux to Sonicated LpA-I***

All the assay conditions were as described in Fig. 4-3. In panel A, the cells were incubated with DMEM containing 45 μg protein/ml of sonicated LpA-I particles with 1.8 moles of UC (S6) and either 11.2 moles of SM (S7) or 6.1 moles of PI (S8) respectively as described in Table 4-II. In panel B, cellular cholesterol efflux to a POPC/apoA-I complex (S3 at 45 μg protein/ml) made from an initial POPC/apoA-I mixture of 20:1 (molar ratio) was compared to the LpA-I complexes (S9 at 45 μg protein/ml) whose half phospholipid content was replaced by sphingomyelin. At indicated time intervals, aliquots of medium were taken for the determination of cellular cholesterol efflux (n=4, +/- SD).


A
S8
S7
S6
DMEM
Medium $^3$H-Cholesterol
( cpm x10$^2$/μg cell protein )
Time ( min )
0
3
6
9
12
0
30
60
90

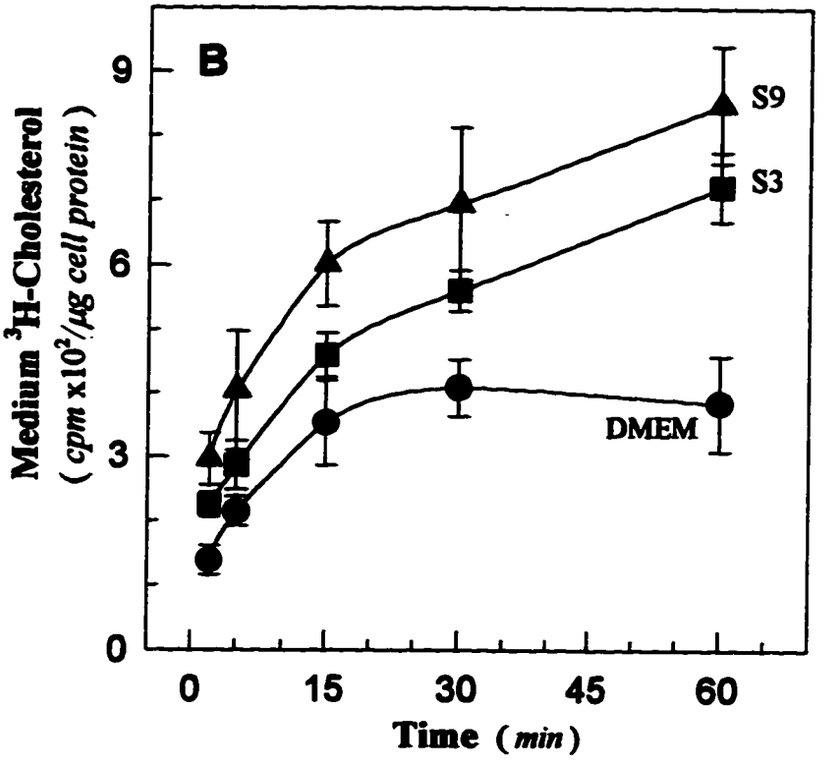

B
9
6
3
0
S9
S3
DMEM
0
15
30
45
60
Time (min)
Medium ³H-Cholesterol
(cpm x10²/μg cell protein)

**Fig. 4-7.** ***Effect of Core Neutral Lipids on Cholesterol Efflux to Sonicated LpA-I.***

All the assay conditions were as described in Fig. 4-3. The labelled cells were incubated with DMEM containing 45 μg protein/ml of sonicated LpA-I particles with 1.8 moles of UC (S6) and either 2.9 moles of cholesteryl esters (S10) or 6.4 moles of triglycerides (S11) or both of them (S12) as indicated in Table 4-II. Aliquots of medium were taken at different time intervals for determination of efflux (n=4, +/- SD).

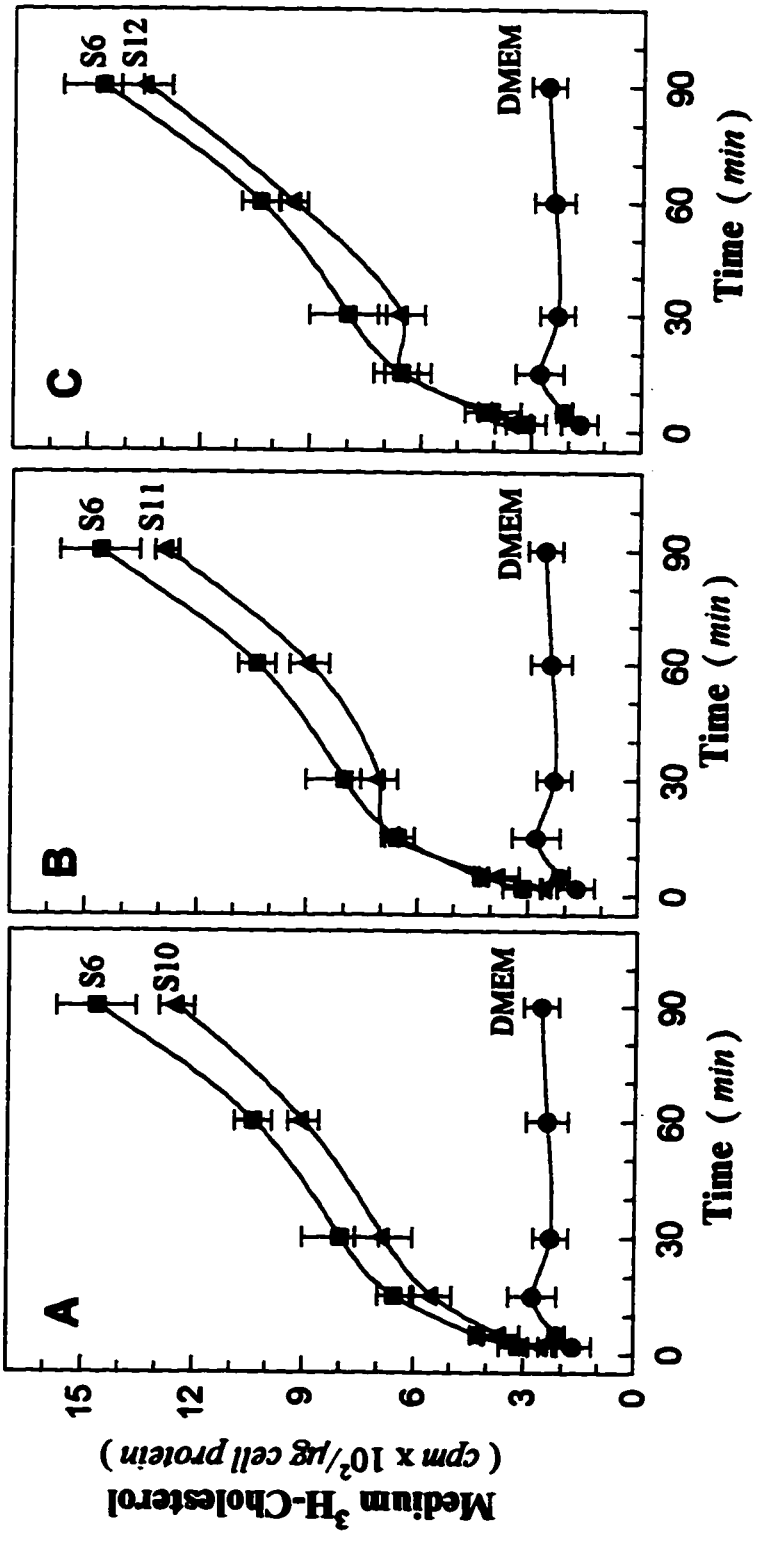

Medium $^3$H-Cholesterol
( cpm x $10^2$/μg cell protein )
15
12
9
6
3
0
A
B
C
S6
S10
S11
S12
DMEM
0
30
60
90
Time ( min )

**Fig. 4-8.** ***Effect of Free Cholesterol on Cellular Cholesterol Efflux to Sonicated LpA-I Particles***

All the assay conditions were as described in Fig. 4-3. The labelled cells were incubated with DMEM containing 45 μg protein/ml of sonicated LpA-I particles with free cholesterol (S6) or without free cholesterol (S5) at the ratio indicated in Table 4-I and 4-II respectively. Aliquots of medium were taken at different incubation time for determination of efflux(n=4, +/- SD).

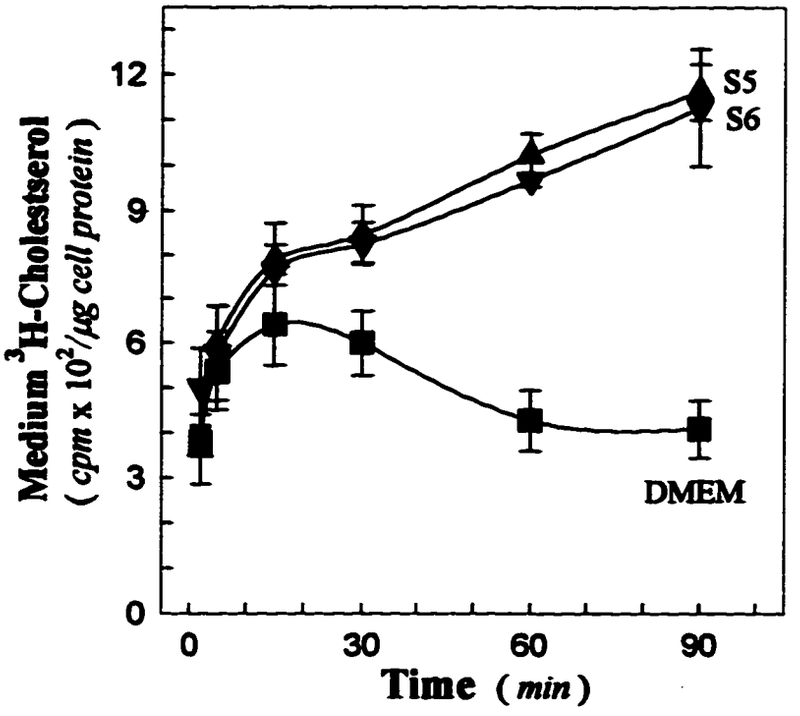
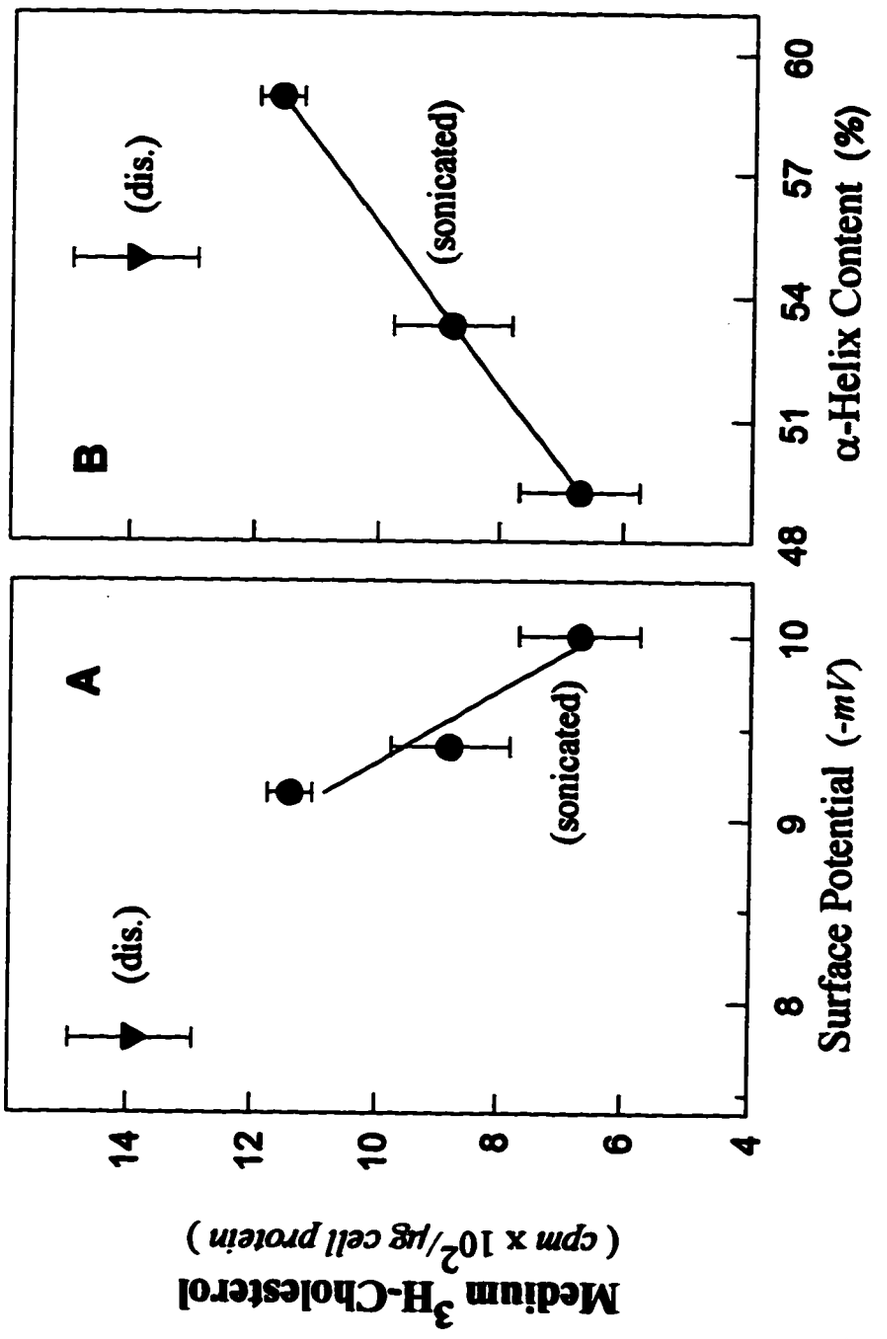

Medium $^3$H-Cholestserol
( cpm x $10^2$/μg cell protein )
12
9
6
3
0
0
30
60
90
Time ( min )
S5
S6
DMEM

**Fig. 4-9.** ***Relationships between the Physical Parameters of Sonicated LpA-I Complexes and Their Abilities to Promote Cellular Cholesterol Efflux***

The regressions between the surface potential, α-helix content of sonicated LpA-I complexes with the abilities of the corresponding complexes to promote cellular cholesterol efflux have been plotted on Panel A and B respectively. the filled circles represent the sonicated LpA-I, while the filled triangles represent a reference discoidal LpA-I (n=4, +/- SD).


Medium $^3$H-Cholesterol
( cpm x $10^2$/μg cell protein )
14
12
10
8
6
4
A
(dis.)
(sonicated)
8
9
10
Surface Potential (-mV)
B
(dis.)
(sonicated)
48
51
54
57
60
α-Helix Content (%)

# GENERAL DISCUSSION

Since Glomset (1968) first presented the hypothesis that HDL plays an important role in RCT, numerous studies have been conducted to characterize the different steps of this process and to identify the factors that determine the efficiency of this pathway. The key step of RCT is the efflux of cholesterol from extrahepatic cells to lipoprotein acceptors, which is determined by the specificity of individual cells and the inherent physico-chemical characteristics of HDL defined by the composition and molecular packing of this lipoprotein.

The purpose of our present study is to identify the composition and related physico-chemical parameters that make HDL avid acceptors for cell-derived cholesterol during a short incubation period. The rationale of this study, as developed in the *INTRODUCTION* of this thesis, is that HDL are present *in vivo* in a great heterogeneity, and that changes in the distribution of HDL subpopulations have been observed in patients with disorder of cholesterol metabolism (Sparks et al., 1989, Williamson et al., 1992, Horowitz et al., 1993, Dobiášová and Frohlich, 1994, Montali et al., 1994). In addition, subspecies of HDL such as pre β-HDL, γ-LpE and LpA-IV have been reported to be the preferential acceptors to access cell-derived cholesterol (Castro and Fielding, 1988, Huang et al., 1994, von Eckardstein et al., 1995). The use of reconstituted HDL-like particles in this study

enables us to overcome the difficulty created by the heterogeneity of native HDL, and makes it possible to investigate the relationships between composition and structure of HDL particles and their function in cellular cholesterol efflux. Although discoidal and spherical HDL are two naturally occurring classes of HDL which can be produced by in vitro reconstitution methods, we have to keep in mind that these are only tentative models of native lipoproteins. Furthermore the reconstitution methods used are obviously irrelevant to the still ill-defined pathways for *in vivo* HDL formation.

1. ***Incubation time and cellular cholesterol efflux***

Different from most of the reported studies, a short term incubation (2 - 90 min) which is more physiologically relevant, has been used for cholesterol efflux experiments in this study. It has been known that interstitial fluid surrounding tissue cells is in a mobile phase, thus a short term incubation simulates more precisely the transient interactions between lipoproteins and peripheral tissue cells in the interstitial space. In addition, short term incubation is able to avoid many events that could have happened in long term incubation. For example, continuous growth and proliferation of cells can lead to an apparent change in cellular cholesterol even under the condition where there is no net efflux of cholesterol to

HDL. In addition, the remodeling/conversion of HDL particles during prolonged incubation may modify the function of the original HDL particles.

## 2. *Serum albumin is a significant mediator of cholesterol transfer between fibroblasts and lipoproteins*

The first question we raised in this study has been whether serum albumin should be included in the culture medium as a saturating protein as many investigators have done. Our preliminary study, as well as others' reports, has clearly showed a significant stimulational function of this protein on cellular cholesterol efflux (Fielding and Moser, 1982, Mendel and Kunitake, 1988, Johnson et al., 1991). Moreover, alterations in lipid and lipoprotein metabolism have been observed both in albumin deficient patients (Cohen et al., 1980, Baldo-Enzi et al., 1987) and in NAR (Nagase et al., 1979, Kikuchgi et al., 1983, Takahaski et al., 1983, Van Tol et al., 1991, and Catanozi et al., 1994). Our detailed analyses for the function of serum albumin in cholesterol transfers in non-cholesterol loaded fibroblasts provide very interesting results. We found that although the binding of albumin to cholesterol is in a low affinity, it appears specific in comparison with the interactions between cholesterol and gelatin or ovalbumin (Fig. 2-4). Serum albumin is not only able to promote the efflux of cellular cholesterol (Fig. 2-1, 2-2) but also able to deliver its cholesterol content to

these cells (Fig. 2-7, 2-8), thus the equilibrium of cholesterol movement between cells and interstitial fluid can be affected by albumin concentration. The presence of lipoproteins which have higher affinity to cholesterol is able to interrupt this equilibrium by attracting the cholesterol from albumin-cholesterol complexes, thus reducing the transfer of cholesterol back to cells (Fig. 2-9). Therefore, albumin can serve as a transient carrier in the process of cholesterol transfer between cells and lipoproteins in extracellular compartment. Our results have been further strengthened with the exclusion of the possible confounding factors, such as apoA-I contamination in serum albumin preparations (Fig. 2-5).

The contribution of human serum albumin and LpA-I in removing cellular cholesterol is less than simply additive (Fig. 2-3), which suggests an interaction between the two cholesterol acceptors. Therefore, we decided to exclude serum albumin in all the subsequent efflux incubations of this study in order to avoid this interaction.

### 3. *Comparison of reconstituted discoidal LpA-I and sonicated "spherical" LpA-I particles upon progressive increase of POPC content*

As mentioned before, discoidal particles are those nascent HDL that have been believed to be the precursors of mature spherical HDL (Glomset et al., 1973). Several subspecies of discoidal lipoproteins has been obtained in *in vitro*

hepatocyte cultures (McCall et al., 1988, 1989, Thrift et al., 1986), in lymph (Reichl et al., 1985, 1990, Sloops et al., 1983a, b, Dory et al., 1985, Forte et al., 1983, Lefevre et al., 1988) and in patients with LCAT deficiency (Soutar et al., 1982, Ohta et al., 1994). However, if, as it has been suggested, HDL can also be assembled from the step-wise lipidation of lipid-free apoA-I, discoidal HDL should be the lipoprotein particles produced secondarily to the small pre $\beta_1$-HDL which has no defined shape and contains exclusively phospholipids and only 1 mole of apoA-I as suggested by Fielding and colleagues (1995a). At present, how the small per β--HDL is converted into discoidal HDL still remains unknown. It has been postulated that the further incorporation of phospholipids and other components into small pre $\beta_1$-LpA-I results in the fusion of the small pre $\beta_1$-HDL particles into the pre $\beta_2$-discoidal HDL (Fielding and Fielding, 1995a).

Our experience in making reconstituted discoidal and spherical particles also supports the feasibility of the apolipoprotein/phospholipid assembly process. When discoidal LpA-I particles are made in the presence of sodium cholate, the minimum POPC level required to make homogenous Lp2A-I is 38 moles per mole apoA-I, which is similar, in a striking contrast, to the maximal POPC level that can be incorporated into a spherical sonicated Lp2A-I (36 moles per mole of apoA-I). In addition, when prepared with a low initial POPC/ApoA-I ratio, the resulting

sonicated LpA-I complexes contains only 1 mole of apoA-I per particle, which may represent the initial precursors in the assembly of HDL. Sonicated spherical Lp2A-I with a size jump to 7.1 nm could be formed upon the initial POPC/apoA-I ratio at or above 20/1 (Table 4-I), suggesting that a progressive increase of phospholipid incorporation could transfer the non-stable small Lp1A-I into relative larger and stable Lp2A-I.

As the sequential POPC incorporation progresses, the physico-chemical parameters of sonicated LpA-I complexes also evolve while remaining distinct from those of discoidal Lp2A-I particles (Table 3-II, 4-I). As mentioned above, the sizes of these sonicated LpA-I range from 5.0-6.0 nm to 7.3 nm, in contrast to those from 9.2 nm to 9.7 nm for discoidal LpA-I. The $\alpha$-helical contents of both particle species increase following the increase of POPC content, however they range from 48.1 to 58.9% for sonicated LpA-I and from 69 to 77 % for discoidal LpA-I. The addition of as little as 2.4 moles of POPC to apoA-I can significantly enhance the negative surface potential to 10.0 mV from 8.3 mV as lipid-free apoA-I. However, further increase in POPC content results in a gradual reduction in surface potential which reaches to 9.2 mV in the presence of 36 moles of POPC per mole apoA-I (Table 4-I). Increasing POPC in discoidal LpA-I particles also has a similar effect on surface potential, which decreases from 7.5 mV to 6.2 mV as POPC content goes from 60 to 133 moles/apoA-I (Table 3-II). The progressive

alterations in the surface potentials and α-helical contents accompanying the gradual increase of POPC suggest that phospholipid may increase the exposure of negatively charged amino acid residues, thus increasing the α-helicity of apoA-I in LpA-I and the stability of these particles (Sparks et al., 1992a, b).

These different but progressive modifications of the physical parameters of these two species of LpA-I particles/complexes may provide an interpretation for their different abilities to release cellular cholesterol as indicated in Fig. 3-5 and Fig. 4-5. At low levels, POPC changes the conformation of apoA-I significantly, probably exposing its amphipathic domains and increasing its potential to interact with cholesterol. Therefore, the abilities of sonicated spherical LpA-I to promote cellular cholesterol efflux is dependent on apoA-I conformation in LpA-I. However, when phospholipids reach a certain level, such as in the range that discoidal LpA-I is formed, the changes in apoA-I conformation are no longer a critical factor for efflux promoting ability of these LpA-I. Instead, the lipid-lipid interaction or phospholipid packing turns to be an important factor in discoidal LpA-I for cholesterol efflux.

## 4. Possible directions for future studies

1) Atherosclerosis is the pathological process defined as the deposition of cholesterol and lipids, and the formation of atherosclerotic plaques in artery walls.

The anti-atherogenic function of HDL is to remove the deposited fat, thereby prevent the formation or promote the regression of the plaques. It has been reported that different cell types display distinct abilities for cholesterol efflux upon exposed to a given species of HDL (Rothblat and Phillips, 1986). Compared to aortic endothelial cells and macrophages, aortic smooth muscle cells are particularly resistant to release their cholesterol content when exposed to HDL or lipid-free apoA-I (Komaba et al., 1992, Li et al., 1993). Therefore, studies aiming at understanding the development and regression of atherosclerotic plaques in vessel wall must be conducted with relevant cells in a relevant context. A model of co-culture of endothelial cells and smooth muscle cells newly established in this laboratory will be a more suitable model for the study of the progression and regression of atherosclerotic process.

2) The present study has shown that the incorporation of the first few molecules of PC into apoA-I may play a critical role in the subsequent attraction of other lipid components to the POPC/apoA-I complexes, which increases the size of LpA-I, and results in the formation of typical HDL particles. Previous studies have provided experimental evidence for the formation of small pre $\beta_1$–LpA-I particles from lipid-free apoA-I (Hara and Yokoyama, 1992, Bielicki et al., 1991, 1992) and for the conversion of discoidal LpA-I into spherical LpA-I (Jonas et al., 1991b, Vanloo et al., 1992); however, process through which the small pre $\beta_1$–

LpA-I fuses to generate the discoidal pre $\beta_2$-LpA-I remains unknown. This question probably can be answered by increasing sufficiently the incubation time to observe the remodeling/conversion of the small POPC/apoA-I complexes.

3) It has been suggested that the structure of the plasma membrane and the distribution of cholesterol in the membrane are the important determinants for the rate of cholesterol efflux. However, although models for the distribution of cholesterol on cell plasma membrane have been postulated (Schroeder et al., 1991, Rothblat et al., 1992, Glaser, 1993), available information is still far from adequate. The relation of cholesterol distribution with the structure of plasma membrane, and the involvement of cell matrix in cholesterol efflux process should be studied.

In addition, it has been shown recently that cholesterol taken up from LDL by fibroblasts accumulated particularly in plasma membrane caveolae in the unesterified form (Fielding and Fielding, 1995c), and that HDL could specifically remove the free cholesterol associated with plasma membrane caveolae (Fielding and Fielding, 1995b). Membrane caveolae have been known to be the mobile cell surface structures enriched in glycosphingolipids, SM and UC, but relatively poor in PC (Brown and Rose, 1992, Hagmann and Fishman, 1982, Smart et al., 1994). Evidence has indicated that caveolae may function in some cells as vehicle for the uptake of small molecules via potocytosis (Anderson, 1992). However, whether or

not these structures are related to the cholesterol-poor and cholesterol-rich domains of plasma membrane suggested by other investigators (Schroeder et al., 1991, Rothblat et al., 1992, Glaser, 1993) still remains unknown. Further investigation on the distribution of free cholesterol in the plasma membrane as well as its relation with plasma membrane caveolae or other cell organelles will provide a better understanding for the mechanism of cholesterol efflux, from which a method for the prevention and the regression of atherosclerosis may eventually be developed.

4) The efflux of phospholipids has been found to accompany the efflux of cholesterol in a number of studies (Hara and Yokoyama, 1992, Bielicki et al., 1991, 1992, Li and Yokoyama, 1993, Li et al., 1995, Yancey et al., 1995). In order to further understand the *in vivo* step-wise assembly process for HDL formation, mechanisms through which these two substrates are released should be examined. It seems impossible that the efflux of both lipids occurs simultaneously and be mediated by a single mechanism since the efflux rates of cholesterol/ phospholipid changed during 24-48 h incubation of mouse macrophages or cholesterol-loaded fibroblasts with lipid-free apoA-I (Yancey et al., 1995, Bielicki et al., 1992). Aortic smooth muscle cells which are significantly resistant to lipid-free apoA-I induced cellular cholesterol efflux have a similar phospholipid efflux rate to that of macrophages (Li et al., 1993). Moreover, a rapid initial release of phospholipid

without efflux of cholesterol has been observed in the incubation of L-cells with apoA-I (Yancey et al., 1995). Our present study has clearly demonstrated that the binding of phospholipid to apoA-I and the resulting apoA-I/phospholipid complexes are the first steps for cholesterol efflux (Fig. 4-5). Several suggestions have been made regarding to the mechanism of phospholipid efflux, which includes i) aqueous diffusion, 2) transient interaction of apolipoprotein with plasma membrane, and 3) the shedding of phospholipid vesicles from phospholipid-rich, cholesterol-poor domains of plasma membrane stimulated by the interaction of apolipoproteins with the cells (Schroeder et al., 1991). Further studies are needed to answer this question.

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# CURRICULUM VITAE

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## Publication:

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**M.Sc. Thesis:**

"The evaluation of zinc and copper nutritional status of adolescent athletes and the effects of exercise on zinc and copper metabolism."

***Abstracts and Presentations:***

1. Zhao, Y., and Marcel, Y. L., Apolipoprotein (apo)A-I and phospholipid contents of reconstituted lipoproteins regulate both sizes and abilities to release cholesterol from fibroblasts. The 68th Scientific Session of American Heart Association, 1995, Anaheim, California, USA.

2. Zhao, Y., and Marcel, Y. L., Phospholipid levels incorporated into apolipoprotein A-I by sonication determine the rate of cellular cholesterol efflux. Canadian Lipoprotein Conference, 1995, Jasper, Alberta, Canada.

3. Zhao, Y., and Marcel, Y. L., Apolipoprotein A-I number and phospholipid species of discoidal LpA-I regulate its ability to promote cholesterol efflux from fibroblasts. The Xth International Symposium on Atherosclerosis, 1994, Halifax, Canada.

4. Zhao, Y., and Marcel, Y. L., Human serum albumin (HSA) is an important contributor to cellular cholesterol transfer. Canadian Lipoprotein Conference, 1994, Halifax, Canada.

5. Frank, P., Bergeron, J., Zhao, Y., Denefle, E., Rassart, E., and Marcel, Y. L. Structural and functional characterization of an apoA-I mutant in reconstituted Lp2A-I. The Molecular Basis of HDL Antiatherogenicity Satellite of the Xth International Symposium on Atherosclerosis, 1994, Halifax, Canada.

6. Zhao, Y., and Chen, J. D. The assessment of zinc and copper nutritional status during endurance training. VIth National Conference of Nutritional Science, 1987, Beijing, China.

7. Zhao, Y., and Chen, J. D., Effects of endurance exercise on the metabolism of zinc and copper. XIIth International Conference of Sports Medicine, 1986, Beijing, China.

***Papers in Refereed Journals:***

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7. Tu, Y. H., Zhu, W. Y., and Zhao Y. W. (1988) Effect of zinc and copper on the protection of somatostatin against islet β-cells damage induced by streptozotocin. Sheng Li Hsueh Pao--Acta Physiologica Sinica 40, 400-405.

8. Zhao, Y. (1988) (review) The exercise induced alteration of zinc and copper metabolism. Sports Science 3, 53-58.

9. Zhao, Y., and Chen. J. D. (1987) Study on the nutritional status and body composition of girl gymnasts during body weight control. Sports Science 2, 78-83.

10. Zhao, Y., and Chen, J. D. (1987) The evaluation of nutritional status of zinc and copper in adolescent athletes. Journal of Chinese Sports Medicine 6, 239-247.

11. Zhao, Y., Li, Yuan., Wang, J., and Liu, X. (1984) Survey of iron-deficient anaemia for pre-school children in Beijing area. Acta of Beijing Medical University 12, 123-128.