special report

International Symposium on Reverse Cholesterol Transport. Report on a Meeting

Andras G. Lacko* and P. Haydn Pritchard†

Texas College of Osteopathic Medicine,* University of North Texas,* and the University of British Columbia†

The International Symposium on Reverse Cholesterol and Coronary Heart Disease was held on November 11–12, 1989 in Fort Worth, TX, sponsored jointly by the Texas College of Osteopathic Medicine and the University of British Columbia. The meeting was endorsed by the American Heart Association and attracted 140 participants including 39 from outside the United States.

PROGRAM

Preparations for the meeting, including the selection of the topics and the speakers, were accomplished with the advice and consent of the investigators experienced in the area of reverse cholesterol transport. The individuals in the advisory groups are: Steering Committee: John J. Albers, Philip J. Barter, Christopher J. Fielding, Alan R. Tall, Peter J. Dolphin, and Paul Roheim; Organizing Committee: Ana Jonas, Hans U. Kloer, G. M. Kostner, Walter J. McConathy, John Oram, and George Rothblat.

Each topic took up the whole length of a morning or an afternoon session. The presentations were organized so that no more than five speakers were involved in any of the sessions in order that ample time was available for a vigorous discussion. These plans were successfully executed and everyone felt that the meeting provided a unique opportunity for each participant to learn about the specific research topics in depth.

PRESENTATIONS

A gratifying aspect of the whole meeting was that many of the speakers presented new findings or novel interpretations of existing findings so that new insights were developed on a number of aspects of reverse cholesterol transport. The concluding summary by Dr. Daniel Steinberg on the direction and the progress of research in reverse cholesterol transport was particularly timely and it is likely to have a lasting impact on future research in this area.

A. HDL metabolism and cholesterol efflux (Chairmen: Drs. Walter J. McConathy and Arie Van Tol)

Dr. George Rothblat led off the first session by presenting an overview on the interaction of the HDL and cellular pools of cholesterol followed by a summary of some of the recent findings from his laboratory. These studies included the investigation of bi-directional free cholesterol flux between HDL and cultured cells. The data showed that depletion of phospholipids from HDL reduces the efflux of cholesterol from transformed cells but not from normal cells. Additional work has revealed that the type of cholesterol acceptor particle (HDL species) did not selectively influence the release of unesterified cholesterol from lysosomes after the hydrolysis of the intracellular cholesteryl ester pool. The presence of an unesterified cholesterol acceptor (HDL) had a marked effect on the mobilization and clearance of esterified cholesterol from peritoneal macrophages; however, specific rat hepatoma cells and transformed mouse peritoneal macrophages only exchange rather than export cholesterol under these conditions. These findings underline the importance of the surface characteristics and other properties of specific cell lines used in studies that probe cell/lipoprotein interactions.

The next speaker, Dr. Jack Oram, introduced an elegant hypothesis for the interaction of HDL with specific cell surface binding sites that appears to result in the production of a secondary message promoting the efflux of intracellular cholesterol to the cell membrane and to acceptor lipoproteins (HDL). Dr. Oram also described the strategy for the cloning of the cDNA of the protein that is involved with HDL binding and reported on the predicted secondary structure and functional domains of this

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; LTP, lipid transfer protein; LCAT, lecithin:cholesterol acyltransferase.

protein. In addition, he presented preliminary data on the expression of the recombinant HDL receptor in BHK cells.

Drs. Steven Kunitake and George Melchior shared the time period allotted for the discussion of pre-beta HDL complexes. Dr. Kunitake reported that the predominantly apoA-I-containing pre-beta HDL particles have a less stable structure than the major (α-migrating) HDL pool as the former are more susceptible to either proteolytic digestion or heat denaturation. Esterification of cholesterol by LCAT was accompanied by movement of apoA-I from the pre-beta to the alpha (HDL) fraction, whereas transfer of cholesteryl esters from HDL to LDL and/or VLDL promoted the movement of apoA-I into the pre-beta fraction.

Dr. Melchior presented data on the secretion of pre-beta-migrating, apoA-I-containing lipoproteins secreted by hepatocytes isolated from nonhuman primates. In these cultures, the pre-beta migrating HDL ($\sim$50 kDa) predominates in the culture medium, especially in the early growth phase of the cells (between days 2 and 6). The alpha HDL ($\sim$100 kDa) begins to accumulate slowly beyond day 6 but the pre-beta HDL remains the predominant apoA-I-containing component. This may be due to the absence of plasma components which normally catalyze the rapid conversion of the pre-beta HDL to the alpha migrating particles.

Dr. Ray Pittman was the last speaker of the first morning session. He reported on the testing of the hypothesis that selective uptake of HDL components (particularly cholesteryl ester (CE) and apoA-I) by specific tissues plays a significant role in reverse cholesterol transport. The rabbit was chosen as the experimental model as this species has about four times the CETP activity of humans. Dr. Pittman reasoned that if selective uptake occurred in the face of such high CETP activity, then it must make a significant contribution to the elimination of cholesterol from the plasma by the liver. The experimental approach included kinetic modeling to account for the exchange rates between lipoprotein classes and four different tracers were used (to label apoA-I and CE of HDL, apoB of LDL, and the CE of VLDL). Analysis of the data showed that in spite of the high rate of CE transfer, 30–40% of the total CE clearance was by selective uptake, suggesting that in humans (where CETP activity is considerably lower) a considerably larger portion of HDL-CE is likely to be processed by this route.

B. Lecithin:cholesterol acyltransferase (Chairmen: Drs. Henry J. Pownall and L. L. Rudel)

This session began by Dr. Christopher Fielding's presentation on LCAT. As Dr. Fielding was not able to attend, Dr. Paul Roheim kindly agreed to read his paper and attempt to answer questions. In addition to the overview on the structure and function of LCAT, three major points emerged from Dr. Fielding's study. First, he observed that the extensive analysis of normal and mutant LCAT genes has so far failed to support the existence of two or more separate genes for distinct species (α and β) of LCAT as had been implied by the studies on the Scandinavian patients with fish-eye disease. Second, certain aspects of the catalytic mechanism for LCAT, proposed by Jauhaieinen and Dolphin, were questioned regarding the specific role of the active site cysteine groups. Dr. Fielding was able to reproduce the earlier findings of Jauhaieinen and Dolphin using sonicated liposomes but not with the French pressure cell-prepared liposomes as substrates. Third, there is an increasing amount of evidence forthcoming from a number of laboratories suggesting that LCAT circulates as part of a noncovalent complex comprised of specific HDL components including apoA-I. This system is the likely recipient of the free cholesterol as soon as it leaves the cell membrane and thus represents the initial phase of reverse cholesterol transport.

The next presentation was given by Dr. Peter Dolphin who reviewed his laboratory's recent contributions to the understanding of the mechanism of action of LCAT. The enzyme reaction is proposed to consist of three sequential steps that are normally concerted within the catalytic site and involve: *1*) phospholipase A_2-like activity (cleavage of the *sn*-2 fatty acid of phosphatidylcholine); *2*) an internal transacylation (transfer of the fatty acyl group from a serine residue to a cysteine); *3*) cholesterol esterification (transfer of the fatty acyl group from the cysteine to the 3-hydroxyl group of cholesterol.

The first step involves Ser-181 and the second and third steps, Cys-31 and Cys-184. The latter two residues have been proposed by Yang et al. (*J. Biol. Chem.* 1987. **262:** 3086–3091) to be brought into close proximity by the two disulfide linkages in the tertiary structure of LCAT). Subsequently, Jauhiainen et al. have shown by the simultaneous chemical modification of both Cys-31 and Cys-184 that these two residues are vicinally located with their sulfur atoms 3.5–3.62 Å apart. Evidence from additional chemical modification studies is consistent with the proposed mechanism including the formation of a Cys-acyl intermediate. During the question period, Dr. Dolphin was asked to interpret Dr. Fielding's finding of being unable to show inhibition of transesterification (as a consequence of blocking the cysteine groups) when liposomes prepared by French pressure cell were used as substrate. Dr. Dolphin stated that he had not used substrates prepared in that manner and responded by reporting some recent data form his laboratory that showed inhibition of cholesterol esterification but not the phospholipase activity after sulfhydryl modification even when intact HDL_3 was used as a substrate. He also noted that Dr. Jonas has recently reported similar results using rat HDL.

Dr. Haydn Pritchard then discussed the function of LCAT using native lipoprotein substrates. Although considerable consensus exists concerning HDL as the major lipid substrate provider for the LCAT reaction, the nature of the enzyme/substrate interaction and the function of the enzyme in some pathological states are far from clear. Dr. Pritchard reviewed the evidence available with normal plasma samples, contrasting these to studies conducted with patients who had HDL deficiency syndromes. In normal plasma and in plasma from patients with LCAT deficiency, Tangier disease, or fish-eye disease, the LCAT associates with an HDL-size lipoprotein particles as judged from gel chromatography, precipitation studies, and immunoaffinity chromatography. This lipoprotein complex contains little if any apoA-I in Tangier disease. An intriguing question is presented by the findings of Holmquist and Carlson who proposed the existence of two enzyme species (α and β LCAT) based on incubation experiments with normal and fish-eye disease plasma samples. It appears that even though the LCAT in fish-eye disease strongly prefers the apoB-containing lipoproteins as substrates, the enzyme still associates with an HDL-size lipoprotein fraction. Two tentative conclusions may be drawn from these studies. First, the size of the lipoprotein complex rather than subtle differences in apolipoprotein or lipid composition appears to be the main determining factor concerning the suitability of lipoproteins as LCAT substrates. Second, the binding of LCAT to a particular lipoprotein species may not be prerequisite for the utilization of lipid substrates from that lipoprotein complex.

The next speaker, Dr. Paul Roheim, discussed the importance of interstitial cholesterol transport, emphasizing that this compartment is three times as large as the plasma compartment. He showed that HDL recovered from interstitial fluid is rich in apoE and apoA-IV and relatively poor in apoA-I compared to plasma HDL. The LCAT activity of interstitial fluid was found to be relatively low, probably due to the poor reactivity of interstitial HDL with LCAT which is likely to result in the relatively high content of unesterified cholesterol in interstitial lipoproteins. The discussion on interstitial lipoproteins was continued by Dr. Michael Lefevre. He further described the heterogeneity of the HDL isolated from lymph. He also presented a hypothesis that changes in HDL structure occur during the filtration process into interstitial spaces. Interaction of the interstitial HDL with unesterified cholesterol from peripheral cells or apoE-containing discoidal HDL appears to result in cholesterol-enriched HDL particles. Experimental evidence for this hypothesis, involving studies with isolated, perfused dog paws, was presented to follow the enrichment of interstitial HDL with unesterified cholesterol.

C. Lipid transfer proteins (LTP) (Chairman Dr. Gerhard Kostner)

Because some investigators prefer the designation CETP, we will use both LTP and CETP to adhere to the same nomenclature that the speakers used in their presentations. As a lead-off speaker, Dr. Yves Marcel provided an overview of our current understanding of the structure and function of CETP and subsequently reported on collaborative studies conducted with Drs. Ruth McPherson and Alan Tall. Dr. Marcel and co-workers have developed a series of monoclonal antibodies (mAbs) that react with CETP. Three of these mAbs inhibited CETP activity and the epitope of one specifically mapped to the C-terminal region. Upon reaction with this latter antibody, the neutral lipid uptake by CETP was blocked, suggesting that the neutral lipid binding site of CETP may be located in the C-terminal region. Additional studies involved the development of a solid phase radioimmunoassay which avoids the interference by lipoproteins in the measurements of CETP levels. Using this new technique, Dr. Marcel and his coworkers were able to show that, in 29 normolipidemic subjects, CETP levels positively correlated with apoA-I, apoE, and HDL cholesterol but not with apoA-II, apoD, or apoB. However, there was only a mild elevation of CETP protein levels (compared to those of normal controls) in patients with familial combined hyperlipidemia or familial hypercholesterolemia. Dr. Marcel also reported that probucol administration resulted in increased CETP mass which has been described to correlate with decreased HDL cholesterol levels.

The next speaker, Dr. Richard Morton, discussed the regulation of the lipid transfer protein (LTP)-mediated activity in the plasma. He identified three factors that regulate LTP activity. *1*) The composition of substrate lipoproteins, particularly their free cholesterol content, plays a key role in determining the rate of lipid transfer. Higher free cholesterol levels result in the enhancement of CE removal from HDL which is expected to enhance LCAT activity. *2*) The biosynthesis and secretion of LTP by cultured cells were inhibited by LDL and stimulated by HDL, suggesting that cellular cholesterol metabolism and LTP secretion may be linked. *3*) A protein with a molecular weight of ~28,000 has been found to inhibit both TG and CE transfer activities of LTP by apparently competing for the lipoprotein binding site of CETP. The inhibitor is more effective against VLDL or LDL than HDL as either donors or acceptors of neutral lipids.

Dr. Robert Phair reported on innovative studies on the characterization of a class of apoB-containing lipoproteins referred to as the "lipid transfer zone." He described experiments that he had jointly carried out with Dr.

Deborah Applebaum-Bowden using density gradient centrifugation combined with Superose chromatography for their isolation and gradient gel electrophoresis for the size determination. The laboratory data confirmed predictions derived from computer models to show that a discrete class of apoB-containing lipoprotein species, with molecular weights between 4.27×10^6 and 5.26×10^6, was particularly effective in accepting cholesteryl esters from HDL. These lipoprotein particles are in the conventional IDL range and are predicted to handle 1.5 mmol cholesterol per day, 18% of the total that is handled by the periphery).

The last speaker of this session, Dr. Lou Agellon, reported on the molecular biology of the CETP gene. He described the sequence and the organization of the CETP gene into 16 exons and 15 introns. The organization of the coding regions and the arrangement of introns does not resemble any of the genomic characteristics of apolipoproteins or lipoprotein-associated enzymes. However, the amino acid sequence in the hydrophobic region of the signal peptide contains a pentapeptide (Val-Leu-Thr-Leu-Ala) that is 100% homologous with a similar region of apoA-I, apoA-IV, and lipoprotein lipase. The homology in the signal region suggests that this pentapeptide sequence may be involved in a specialized transport of these polypeptides during the secretory process. A study of a Japanese family with CETP defficiency revealed that the absence of circulating CETP was due to a primary defect involving a G→A mutation in the splice signal region of the gene producing the change 5'-GT-3'→5'-AT-3'' resulting in an abnormally large HDL pool. Studies with rabbits have shown that both the circulating CETP levels as well as the amount of liver mRNA coding for CETP increased upon cholesterol feeding.

D. Clinical aspects of reverse cholesterol transport (Chairman: Dr. Jiri Frohlich)

Dr. Ernest J. Schaefer discussed the role of HDL in coronary heart disease. He pointed out that even though the turnover of the protein component is relatively slow, the half life of the cholesterol component is quite short. Studies on the regulation of HDL levels in human subjects revealed that the decreased production rate rather than the hypercatabolism of apoA-I may be the more significant factor in the hypoalphalipoproteinemia which predisposes to atherosclerosis. For instance, absolute synthetic defects (apoA-I and apoA-I/C-III deficiencies) are associated with premature atherosclerosis. Conversely, HDL deficiency due to hypercatabolism does not nearly present the same degree of risk. Interestingly, based on recent studies, the absence of apoA-II does not influence either the level of apoA-I or the level of HDL cholesterol and thus poses no risk of coronary heart disease. Attempts to associate polymorphism in the gene cluster of A-I/C-III/A-IV have so far failed to reveal effective risk indicators. Consequently, the levels of apoA-I and HDL cholesterol continue to be the most useful parameters to monitor cardiovascular risk. For example, the HDL levels are below the 10th percentile in 45% of the patients with coronary heart disease. However, low HDL levels are likely to be related to other lipid disorders (hypertriglyceridemia) because the incidence of isolated hypoalphalipoproteinemia in pateints with coronary artery diseases is quite low (below 5%). Overall, low HDL-cholesterol level continues to be the most powerful indicator of coronary heart disease within a population and raising HDL levels by only 1% may produce a 2% decrease in coronary risk.

The next speaker, Dr. Cesare Sirtori, reviewed recent studies concerning the effects of a number of therapeutic approaches including hypolipidemic agents on components of reverse cholesterol transport. He found relatively few changes upon the administration of a nicotinic acid derivative (acipimox), the only potential effect being the inhibition of hepatic triglyceride lipase. Bezafibrate administration to normolipidemic subjects resulted in decrease of HDL_{2b} levels without effects on LCAT or CETP activities. Probucol lowered both LDL and HDL levels; the resultant HDL particles are mainly HDL_3 with almost complete loss of HDL_2. The observed changes in mass and size distribution of HDL are with increased reverse cholesteryl ester transport. Dr. Sirtori drew three major conclusions from his studies: *1*) the transfer of HDL-CE to LDL is protective; *2*) this transfer process may be reduced by alcohol intake, diabetes, hypertriglyceridemia, and uremia and it may be increased by probucol; and *3*) the absence of CE transfer may result in hyperalphalipoproteinemia.

The last speaker, Dr. Daniel Steinberg, provided a summary of the discussions that transpired at the meeting and also made some insightful comments regarding the future directions of the research efforts addressing the role of HDL in coronary heart disease. While he acknowledged that the clinical data were overwhelmingly in favor of HDL measurments being the best index of coronary risk, the involvement of HDL molecules protecting the arteries or reversing atherosclerosis is unclear. In order to develop the necessary information for a better understanding of the role of HDL in the prevention of coronary heart disease, Dr. Steinberg suggested that direct experimental evidence is needed from studies with experimental animals or humans to show that changes in the levels of HDL (or HDL subfractions) indeed influence the development of arterial lesions.

Regarding the information presented at the meeting, Dr. Steinberg noted that the best current hypothesis for the interpretation of the epidemiological data is provided by reverse cholesterol transport; however, there are many remaining concerns. For instance, during the studies of reverse cholesterol transport, the amount of cholesterol

transported is estimated for the whole body or between specific organ systems. These calculations are unable to predict the amount of cholesterol entering or leaving the arteries. Consequently, experimental models are needed where the flux of cholesterol in arterial tissue can be directly estimated.

Dr. Steinberg also offered a number of potential alternatives to reverse cholesterol transport that may explain the role of HDL in retarding or reversing atherogenesis. These included: the competition of apoE-rich HDL with LDL for hepatic receptor sites, the protection of LDL from oxidation, and the inhibition of LDL aggregation prior to foam cell formation. In addition, Dr. Steinberg also noted that the high risk condition projected by low HDL levels may be only a secondary indicator of abnormalities in triglyceride metabolism, including high plasma triglyceride levels perhaps due to decreased lipoprotein lipase activity. Finally, the quantification of reverse cholesterol transport will be necessary in vivo in order to determine whether it is a major mechanism responsible for the antiatherogenic potential of HDL.

SUMMARY

The purpose of this symposium was to provide a forum for the reporting of recent findings and the exchange of ideas concerning reverse cholesterol transport, an area of intense interest and some controversy. Data from epidemiological studies have consistently shown that elevated levels of high density lipoproteins (HDL) are an index of increased protection against coronary heart disease. However, the mechanism whereby HDL is involved in the prevention and/or reversal of atherosclerosis is unknown. According to one of the hypotheses, HDL acts as the primary acceptor of unesterified cholesterol from cells and functions jointly with the enzyme lecithin:cholesterol acyltransferase (LCAT) and the cholesteryl ester transfer protein (CETP) to facilitate the movement of cholesterol from peripheral tissues to the plasma and ultimately to the liver. Although this mechanism as originally proposed by Glomset is an essential physiological mechanism, the clincial significance of this hypothesis remains unsubstantiated. Key elements of knowledge are lacking that would allow the linking of cholesterol efflux from cells and tissues with specific events in HDL metabolism, particularly those that are relevant to the prevention and/or reversal of atherosclerosis. Because of the intricate nature of the interaction between the components of reverse cholesterol transport, a conference involving the leading investigators of the field, where extensive discussion of the findings and ideas is allowed, appeared highly desirable. Indeed, from the distance of nearly 4 months, feedback from the participants indicates that the meeting was highly successful and the organizers feel that all the projected goals of the symposium were accomplished.

We are grateful for the assistance of Cindy Azuma, Dr. M. K. Jacobson, and Jan Sharp with the organization and local arrangements, and to the Amon Carter Foundation, Team Bank, trust division, Bristol Meyers, The Osteopathic Medical Center of Texas, Health Care of Texas, Warner Lambert/Parke Davis of Canada Inc., Squibb & Sons, Sandoz Pharmaceuticals, The Upjohn Company, Merck Sharp and Dohme, the Texas Osteopathic Medical Association, and Key Pharmaceuticals for their financial support.