special report

1993 Aspen Cholesterol/Bile Acid Conference: Diet and gene interactions in cholesterol metabolism

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The 1993 Aspen Cholesterol/Bile Acid Conference "Diet and Gene Interactions in Cholesterol Metabolism" was held at the Given Institute of the University of Colorado School of Medicine in Aspen, Colorado on August 21–24, 1993.

Dr. Nobuyo Maeda reported on dietary responses of mutant mice generated by gene knockout procedures. In inbred mice (strain 129) modified to lack apoA-I, there was a reduction in total plasma cholesterol and HDL cholesterol of almost 70% and 80%, respectively. No atherosclerosis was present in mutant mice maintained on a chow diet. However, in mutant mice fed a diet rich in fat (16% saturated fat) and cholesterol (1.25%) a 20–30 mg/dl increase in total plasma cholesterol resulted, while plasma cholesterol in normal litter mates increased by 60 mg/dl. Early signs of atherosclerotic lesions (i.e., small areas of fat deposition) were observed after 20 weeks of feeding the atherogenic diet, but the incidence or extent of fat deposition was not related to the apoA-I phenotype. The results imply that a deficiency in apoA-I did not by itself result in atherosclerosis even when mice lacking this apolipoprotein were fed a high fat/high cholesterol diet.

In comparison, mice genetically modified to lack apoE had five times the normal plasma cholesterol concentration and by 3 months of age developed foam cell-rich deposits in the proximal aorta even when fed a regular low fat/low cholesterol mouse chow. By 10 months of age, lesions were widely distributed throughout the arterial tree including the coronary, carotid, and iliac arteries. More advanced arterial lesions showed increasing complexity with many of the characteristics of advanced atherosclerotic lesions in humans such as fibrous caps, cholesterol clefts, calcified deposits, and microthrombi. When homozygotes lacking apoE were fed a fat- and cholesterol-enriched diet, plasma cholesterol concentrations became massively elevated to about 3300 mg/dl and massive depositions of lipid in the aorta was observed after only 3 months. In heterozygotes fed the high fat/high cholesterol diet for the same time periods, plasma cholesterol concentrations were elevated to approximately 350 mg/dl and atherosclerotic lesions were observed in the proximal aorta. In contrast, in normal litter mates fed the same diet, no atherosclerotic lesions were present even though the plasma cholesterol levels were about 250 mg/dl. Although heterozygotes did not develop lesions on a regular chow diet, the results indicated that apoE deficiency was a risk factor for the development of diet-induced atherosclerosis in these mice.

Dr. Jean MacCluer discussed genotype by diet interactions in nonhuman primate models, and addressed the advantages that studies in animal models have over studies in humans for controlling diet and other environmental variables. Any individual's genotype influences his/her response to an atherogenic diet, and it is important to understand gene/diet interactions because of the implications for intervention. Using pedigreed baboons to determine familial patterns of variation in response to different diets, it is possible to demonstrate that a specific gene locus (loci) can be influenced by components of the diet and result in specific changes in serum levels of lipoproteins and apolipoproteins. Known candidate genes, and new genes (termed major genes) whose identities and chromosomal locations are as yet unknown, can alter responsiveness to dietary ingredients. Major genes can be detected through statistical means by using complex segregational analysis.

The VLDL+LDL cholesterol response to high cholesterol/saturated fat (HCSF) diets varied according to the genotype for an RFLP at the LDL receptor gene locus. Homozygous animals for the most common allele showed a greater response to dietary challenge than did animals with the other two genotypes. Three loci (for as yet uncharacterized major genes) influenced changes in plasma HDL_1 cholesterol and apoA-I concentrations in response to the HCSF diet. The pattern of variation in HDL_1 cholesterol indicated the presence of at least one major gene, with an uncommon allele that caused an increase on the chow diet and a further increase on the HCSF diet.

The unidentified major locus accounted for 24% of the variance in HDL_1 on the chow diet and 51% on the HCSF diet. At least two additional loci act additively to determine the apoA-I levels. One was responsive to both chow and HCSF diets but primarily the latter, and the second was apparent for the chow diet alone. Complex segregation analyses have supported the interaction between diet and genotype for both major loci. Most genotypes showed an increase in apoA-I response to dietary challenge, but one genotype showed a decrease and the relative ranks of the genotypes changed on the HCSF diet. Additional analyses on animals fed a diet high in saturated fat but low in cholesterol indicated that different genes determine responses to saturated fat and dietary cholesterol. The results of complex segregation analyses therefore have provided a genetic basis for molecular, biochemical, and metabolic studies to identify major genes and document their function in the regulation of lipoprotein metabolism as influenced by diet.

Dr. Aldons J. Lusis reviewed his research involving genetic and dietary interactions and their potential roles in the development of atherosclerosis in inbred mouse strains. C57/BL6 and BALB/C strains have been particularly useful for evaluating the involvement of certain traits as these two strains are susceptible to the formation of atherosclerotic lesions. A number of different traits were discussed for having potential roles in a cascade of events leading to atherosclerotic disease. The following traits were included: *1)* enzymes of cholesterol metabolism (HMG-CoA reductase, cholesterol 7-α hydroxylase); *2)* plasma lipoproteins; *3)* aortic fatty streaks; *4)* arterial calcification (BXH-11); *5)* arterial lipofuscin deposition; *6)* vasculitis and autoimmune disease (MRL/LPR); and *7)* lipid oxidation/inflammation (for example, Ath-1, adhesion molecules, M-CSF, MCP-1/JE, IL-1β, serum amyloid A). The influence of a high cholesterol/high fat diet with or without bile acids on the expression of some of these genes and their impact on atherosclerotic disease was discussed. A hypothesis for atherogenesis was presented that underlies selection of these traits: with elevated plasma LDL levels, some LDL may become trapped in the subendothelial space. LDL lipids may then be modified which, in turn, may stimulate the expression of monocyte-specific adhesion molecules and chemotactic factors. Induced monocyte migration is followed by differentiation into macrophages. Production of highly reactive oxygen species by macrophages may lead to further oxidation of LDL with subsequent unregulated uptake by macrophages resulting in eventual foam cell formation. Growth factors released from foam cells may induce phenotypic differentiation of smooth muscle cells for migration and proliferation within the subintima.

Dr. John Dietschy presented his findings on the metabolic differences associated with hyper- and hyporesponsiveness in cynomolgus monkeys. Studies evaluating cholesterol synthesis and LDL cholesterol transport in this species were presented and compared with those in other animal models. Whole body cholesterol synthesis in cynomolgus monkeys was approximately 10–14 mg/day per kg body weight and fell into the "main-stream" relationship among species where whole body cholesterol synthesis decreased by about 10 mg/day for each 10-fold increase in body weight. LDL cholesterol turnover also fell into the "main-stream" pattern with clearance being approximately 1.0–1.5 pools/day with 70–80% being removed from the blood by the liver.

A group of approximately 100 monkeys were challenged for 4 months with a standardized diet containing saturated fat and cholesterol, and selection of groups of hypo- and hyper-responders to the diet was made by identifying the upper 25% and lower 25% of the response curve. When these animals were fed a low cholesterol diet, hepatic cholesterol synthesis was 4-fold higher in the hypo-responders as compared to the hyperresponders while extrahepatic synthesis was no different. When a high cholesterol diet was fed, plasma cholesterol was elevated to 180 mg/dl in the hyporesponders, while the hyperresponders increased to over 400 mg/dl. However, in both groups the level of hepatic LDL cholesterol transport was appropriate for the level of hepatic cholesterol synthesis and hepatic cholesteryl ester concentrations. Thus, the difference between the two groups was apparently a difference in the net delivery of sterol to the liver by an amount equal to 1.1 mg/day per kg, which is about 10% of the daily total body cholesterol synthesis as measured in this species.

As there was no difference between groups in the rate of extrahepatic cholesterol synthesis, the difference in net sterol delivery to the liver most likely resulted from excessive cholesterol absorption in the hyperresponders, or alternatively, from higher amounts of sterol secreted into the bile and excreted in the feces of the hyporesponding animals. Available data indicated no difference in bile acid pool size or in the types of bile acids in the small intestinal contents of representative animals from the two groups. Speculation was that differences in intestinal cholesterol absorption may account for the difference in diet responsiveness. Different cholesterol absorption rates may result from specific differences in the unstirred water layer resistance, membrane permeability, intestinal ACAT activity, or in processes of chylomicron assembly and secretion.

Studies on gene therapy in familial hypercholesterolemia (FH) were discussed by **Dr. James Wilson.** This disease has emerged as an important paradigm for the development of liver-directed gene therapies. The deficiency in LDL receptors characterizing this disease leads to abnormally high accumulations of LDL cholesterol in blood and premature coronary artery disease. The homozygous form of FH is refractory to conventional therapies, how-

ever, metabolic cure has been achieved by orthotopic liver transplantation. This suggests that an alternative form of therapy based on genetic reconstitution of hepatic LDL receptor by somatic gene transfer may be useful. This requires the development of a delivery vehicle capable of targeting normal LDL receptor genes to hepatocytes in vivo. One approach is to construct synthetic complexes of protein and DNA that are recognized by the hepatocyte-specific asialoglycoprotein receptor. Infusion of these constructs into the blood of WHHL rabbits has resulted in targeted gene delivery and improvement in hypercholesterolemia. This technology, at present, is of limited clinical utility because of inefficient recombinant gene expression that is transient.

A more encouraging technology is the use of recombinant adenoviruses for liver-directed gene therapy. When introduced into the peripheral circulation, these viruses specifically target hepatocytes. Recombinant adenoviruses that express the human LDL receptor gene have been injected into WHHL rabbits. Expression of the recombinant gene is extremely high in liver, leading to normalization of serum cholesterol. The effect, however, is transient and the animals develop an immune response that prevents another administration of virus. A more complex but potentially useful approach to gene therapy of FH is based on transplantation of autologous cells. This approach, called ex vivo gene therapy, involves resection of a portion of liver. Primary cultures of hepatocytes that are genetically corrected with recombinant retroviruses are then established, harvested, and reinfused into the portal circulation. Preclinical studies in WHHL rabbits indicate that ex vivo gene therapy could affect a partial and prolonged improvement in hypercholesterolemia, and subsequent studies in nonhuman primates have confirmed the safety of this procedure. As a result, a clinical trial has begun to treat homozygous FH patients with ex vivo gene therapy.

Dr. James Metherall reported on the isolation and characterization of a mutant Chinese hamster ovary (CHO) cell line (SRD-7) that has defects both in the utilization of LDL-derived cholesterol and in cholesterol biosynthesis. SRD-7 cells were isolated based on their resistance to amphotericin B in the presence of LDL. LDL-derived cholesterol failed to stimulate cholesterol esterification in SRD-7 cells, but esterification rates were normal when sterols were added directly to the culture medium. Results suggest that receptor-mediated endocytosis of LDL is normal in SRD-7 cells, at least to the point of delivering cholesterol to the lysosome. SRD-7 cells also failed to convert lanosterol to cholesterol, a conversion normally occurring at the endoplasmic reticulum (ER).

Treatment of wild-type cells with progesterone inhibited utilization of LDL-derived cholesterol and inhibited cholesterol biosynthesis. Esterification of LDL-derived cholesterol was inhibited 10-fold by 10 μM progesterone. The data suggest that progesterone does not directly inhibit acyl CoA:cholesterol acyltransferase but rather limits the availability of substrate cholesterol to the enzyme. Progesterone also inhibits the conversion of lanosterol to cholesterol. The phenotype of progesterone-treated wild-type cells therefore mimics that of SRD-7 cells. As cholesterol esterification and conversion of lanosterol to cholesterol both occur in the ER, progesterone appears to inhibit the transport of sterols to the ER and SRD-7 cells may have a genetic defect that prevents this transport.

Inhibition of cholesterol esterification by progesterone appeared not to involve the progesterone receptor. A number of other steroid hormones also inhibited esterification, including pregenolone and dehydroepiandrosterone (DHEA). A direct correlation was observed between the ability to inhibit esterification and the general hydrophobicity of the steroid. This pattern of steroid inhibition has also been seen for members of the P-glycoprotein (P-gp) family of membrane pumps, integral membrane proteins that pump amphiphilic substances out of the cell. Normal physiologic function of the P-gp pumps has not been described, but a direct correlation between the ability of a steroid hormone to inhibit esterification and its ability to inhibit P-gp activity was found. Nonsteroidal inhibitors of P-gp (verapamil and Triton X-100) also inhibited cholesterol esterification. Therefore, a member(s) of the P-gp family of proteins may play a direct role in cholesterol metabolism by allowing lanosterol and cholesterol to be transported to the ER for conversion to cholesterol and cholesteryl esters, respectively.

Dr. Skadrite Krisans presented evidence that peroxisomes play an important role in isoprenoid synthesis and cholesterol metabolism. Peroxisomes are an organelle present in all mammalian cells other than mature erythrocytes. Peroxisomes are particularly abundant in hepatocytes and proximal tubular epithelium and in tissues that specialize in lipid metabolism, including sebaceous glands, brown fat, and the nervous system. There is considerable evidence that peroxisomes not only have a role in the oxidation of cholesterol during production of bile acids but also in cholesterol biosynthesis. Rat liver peroxisomes contain enzymes of the isoprenoid synthesis pathway and peroxisomes have been shown to contain acetoacetyl-CoA thiolase, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and more recently, mevalonate kinase, which may be present exclusively in this organelle. The largest concentration of sterol carrier protein 2 (SCP-2) is also found in rat and human peroxisomes. Apolipoprotein E is also found in rat hepatic peroxisomes. Moreover, cholesterol biosynthetic capacity is severely impaired in cultured skin fibroblasts obtained from patients with peroxisomal deficiency diseases. The finding of an apparent "duplicate" enzyme system (i.e., for cholesterol and dolichol synthesis) in the cell is not unique. However, when dual

localization of enzyme activities has been demonstrated, a distinctly different function for each pathway has been found. For example, there are two distinct pathways for the production of HMG-CoA, cytosolic and mitochondrial pathways. The majority of the cell's HMG-CoA is produced in the mitochondria and is converted to ketone bodies, whereas the smaller proportion of HMG-CoA that is produced in the cytosol is used for mevalonate and isoprenoid synthesis. Overall, the findings support the likelihood that peroxisomes play an essential role in isoprenoid biosynthesis and in cholesterol metabolism.

Dr. Ta Yuan Chang reported on the molecular cloning and functional expression of human acyl coenzyme A:cholesterol acyltransferase (ACAT). Accumulation of cholesteryl esters as cytoplasmic lipid droplets within macrophages and smooth muscle cells is a characteristic feature of early lesions of atherosclerotic plaque. Intracellularly, an essential element in forming cholesteryl ester from cholesterol is the ACAT enzyme, a membrane protein located in the endoplasmic reticulum. In spite of the central role of this enzyme in cholesteryl ester metabolism, the ACAT protein has never been purified to homogeneity, and no antibodies directed against ACAT have been reported. This laboratory had previously reported the isolation of Chinese hamster ovary cells expressing human ACAT activity. From the DNAs of these cells, a 1.2-kb exonic human genomic DNA has been cloned. This led to the eventual cloning of a 4-kb cDNA clone (K1) from a human macrophage cDNA library. Transfection of K1 into ACAT-deficient mutant Chinese hamster ovary cells complemented the mutant defect and resulted in the expression of human ACAT activity. K1 contained an open reading frame of 1650 bp encoding an integral membrane protein of 550 amino acids. The cDNA reported here should facilitate future molecular studies on ACAT.

Dr. Richard Anderson discussed the mutations he has recently discovered in the human lysosomal acid lipase/cholesteryl ester hydrolase (HLAL) of cells from humans with Wolman disease (WD), a cholesteryl ester storage disease. This enzyme is crucial for the intracellular hydrolysis of cholesteryl esters and triglycerides, and consequences of its deficiency are seen in the intralysosomal accumulation of cholesteryl ester. The disease is autosomal recessive and is lethal within the first year of life. Although results have suggested that Wolman disease arises from defects at the structural gene locus for HLAL, until this study there has been no direct proof of this. The HLAL gene from a human genomic library was isolated and the clone was used to identify mutations in HLAL alleles from a patient diagnosed to have WD. The HLAL genomic locus consists of 10 exons spread over 36 kb with a GC-rich 5' flanking region characteristic of a "housekeeping" gene promoter. After Southern blotting to establish that the genomic DNA from the patient's fibroblasts had no major rearrangements at the HLAL locus, direct sequencing of the HLAL exons was undertaken. A different mutation was identified in each of the two alleles. One mutation involves the insertion of a "T" residue after position 634 that results in the appearance of an in-frame translation stop signal 13 codons downstream. The second mutation is a "T" to "C" transition at nucleotide 638 with the result that a proline rather than a leucine is now coded for at amino acid 179; this is predicated to lead to the disruption of the α-helical structure in a conserved region of the protein. These mutations are each capable of completely disrupting the catalytic function of the lysosomal acid cholesteryl hydrolase and their presence can account for the extreme phenotype of WD manifested in the patient.

Dr. Alan Tall discussed the genetic deficiency in humans of cholesteryl ester transfer protein (CETP). The first mutation discovered in the CETP gene was a gene splicing defect, due to a G for A substitution in the first position of the fourteenth intron of the CETP gene. Homozygotes with the splicing defect have no detectable plasma CETP and have markedly elevated HDL cholesterol levels (3–4 times normal levels). Heterozygotes with the splicing defect have about 60% of normal levels of CETP activity and mass and have only slightly increased HDL cholesterol levels. Recently, a new CETP gene mutation has been discovered. This involves a missense mutation altering amino acid 442 (from aspartate to glycine) of the 476 amino acid CETP gene. Two probands were discovered, one in Osaka (by Dr. Matsuzawa and colleagues) and one in Chiba City (by Dr. Saito and colleagues). Both of the probands were heterozygous for the CETP missense mutation, with one mutant allele and one normal allele. Despite heterozygosity, HDL levels were markedly elevated and plasma CETP activity was greatly decreased (to about 30% of normal levels).

Expression of a mutant CETP cDNA containing the missense mutation showed about 30% of the activity of wild type cDNA (measured as CETP activity accumulating in media of transiently transfected Cos cells). This finding shows that the mutation is significant but does not readily explain the phenotype in the patients. To investigate possible dominance of the CETP missense mutation, mutant cDNA was co-transfected with wild type cDNA. This resulted in a dominant inactivation of the product of the wild type cDNA. This inactivation appeared to involve both decreased secretion of wild type protein and decreased specific activity of the wild type protein accumulating in media, both as a result of co-expression with the mutant protein. The in vitro results seem to explain the phenotype in the patients, and suggest that the missense mutation may have dominant negative effects on the expression of the wild type CETP allele in vivo. Although the basis of this dominant negative action is still poorly understood, one possible interpretation is that hetero-dimers are forming between the wild type and mutant CETPs. These data suggest the possibility that

dimerization might be a component of the mechanism of neutral lipid transfer mediated by CETP.

Dr. George Melchior presented data on the expression of simian CETP in C57BL/6 mice with a resulting reduction of HDL levels. Studies were described to determine how CETP overexpression caused the HDL reduction. Chemical analysis showed that the HDL of the CETP transgenic mice had about twice as much triglyceride and only about 60% as much cholesteryl ester as the HDL from the wildtype C57BL/6 mice. Because both strains of mice had high levels of a circulating lipase, the triglycerides in the HDL were hydrolyzed when plasma from the mice was incubated at 37°C for several hours. Although the degree of hydrolysis was about the same in both types of HDL, apoA-I was shed from the CETP mouse HDL at a rate 4–5 times faster than it was shed from the C57BL/6 HDL. As a result, the average life-span of apoA-I was significantly shorter in the CETP transgenic mice than it was in C57BL/6 mice. It was concluded that, because CETP expression enriched the core of the HDL with triglyceride, it rendered the HDL more vulnerable to lipolysis, which shortened its plasma residence time. That, in turn, led to lower plasma HDL levels.

The second part of Dr. Melchior's presentation described the effects of CETP gene expression on the lipoprotein profile of mice fed a high-fat, high-cholesterol diet as previously described. Briefly, those studies showed that the VLDL+LDL levels increased to a much greater extent in atherogenic diet-fed CETP transgenic mice than it did in the diet-fed controls, and therefore the (VLDL+LDL)/HDL ratio was markedly higher in the transgenic mice. In association, the CETP transgenic mice had more extensive atherosclerosis than did the C57BL/6 controls after 28 weeks on diet.

Dr. Mark Doolittle reviewed his work on the determinants of lipase posttranslational processing. To understand factors controlling the expression of lipoprotein lipase (LPL) and hepatic lipase (HL) Dr. Doolittle's group has studied early posttranslational processes that have been implicated in the conversion of an inactive "precursor" lipase to an active enzyme. These factors include N-linked glycan processing occurring within the ER and Golgi, and a mutation in mice called combined lipase deficiency (cld). Cells homozygous for the cld mutation synthesize LPL and HL normally, but the lipases are retained intracellularly as inactive proteins (the cld mutation and the lipase structural genes are on separate chromosomes). Results indicate that only the first step in N-linked glycan processing, glucose trimming, is essential to the formation of a completely active lipase. Later glycan processing events in the ER and Golgi are not required, and LPL engineered to contain the ER-retention signal "KDEL" is completely active. Studies of LPL and HL processing in cells homozygous for the cld mutation have also shown that LPL and HL are retained within the ER as unprocessed, high-mannose forms. Treatment o cells from homozygotes with brefeldin A causes the redistribution of the Golgi processing enzymes back to the ER resulting in the conversion of high-mannose LPL to the complex form; nevertheless, the fully processed lipase is still inactive. Thus, the inability of LPL to attain enzymatic activity within the ER of cld/cld cells does not appear to be related to glycan processing. Dr. Doolittle hypothesized that the cld mutation must affect a step that, although independent of glycosylation, is essential to the formation of newly synthesized LPL and HL precursors into active conformations.

Dr. Michael Pape reviewed work aimed at characterizing the in vivo and in vitro expression of genes whose products are involved in the regulation of lipid and lipoprotein metabolism in the rabbit. Partial cDNAs for 13 of these genes have been cloned. These include: cholesteryl ester transfer protein, apoA-I, apoB, apoE, LDL receptor, HMG-CoA reductase, cholesterol 7α-hydroxylase, hepatic lipase, lipoprotein lipase, albumin, LCAT, cholesterol ester hydrolase, and PAI-1. mRNA levels for several of these genes in rabbit parenchymal cells in culture were assayed. Surprisingly, apoA-I mRNA levels in cultured parenchymal cells were approximately 50-fold higher than in intact liver after 48 h in culture; LDL receptor and HMG-CoA reductase mRNA levels were also elevated but to a much smaller degree, while levels of other analyzed mRNAs were lower or the same as those levels in intact liver. In order to determine what was responsible for the high apoA-I mRNA levels in cultured cells compared to intact liver, various media components (serum, exogenously added media factors, etc.) were analyzed for their effect on apoA-I mRNA levels. Under all culture conditions tested, apoA-I mRNA levels were elevated at least 25-fold compared to liver levels, suggesting that a media factor was not a major factor in the apoA-I mRNA elevation.

Dr. Pape hypothesized that nonparenchymal cells secrete a factor that inhibits apoA-I mRNA expression. Conditioned media from nonparenchymal cells inhibited the elevation of apoA-I mRNA in a dose-dependent and reversible fashion while there were only slight changes in the levels of apoB, apoE, and hepatic lipase mRNAs. Changes in mRNA levels corresponded to altered apoA-I synthesis/secretion. The inhibitory factor was >30 kDa and was active after heating at 70°C and after exposure to protein denaturing concentrations of urea followed by dialysis. The data suggest that, in rabbits, hepatic parencymal-nonparenchymal communication in the form of soluble factor may be responsible for the low levels of apoA-I mRNA extant in liver.

Dr. Helen Hobbs gave a brief overview of the basic biochemical, physiological, and epidemiological properties of Lp[a] before concentrating on its genetics. The apo[a] gene resides on chromosome 6 next to the plas-

minogen gene which it resembles in structure and sequence. The apo[a] gene is distinguished by the presence of multiple tandem copies of a 5.5 kb sequence that encodes a cysteine-rich motif resembling kringle 4 of plasminogen. Only a subset of the repeats, the so-called type A and type B repeats, are nearly identical in sequence and these are the repeats that vary in number between alleles giving rise to the length variation responsible for the remarkable size heterogeneity of the apo[a] protein; there are at least 34 apo[a] isoforms of different size. Family studies disclosed that the apo[a] gene, or sequences closely linked to the gene, account for approximately 90% of the inter-individual variations in plasma Lp[a] levels.

The structure of the apo[a] gene, and the size and quantity of circulating apo[a] glycoprotein were compared in Caucasians, Chinese, and African-Americans. In both Caucasians and Chinese, the plasma levels of Lp[a] were highly skewed towards lower levels, whereas in the African-Americans the distribution of plasma levels is more Gaussian in appearance. In general, an inverse relationship between apo[a] isoform size and plasma concentration of Lp[a] occurs in all three populations. However, exceptions to this overall trend were numerous, especially among individuals with apo[a] isoforms of smaller sizes.

The distributions of apo[a] allele sizes, as assessed by pulsed field gel electrophoresis and genomic blotting, were compared in the three populations. As reported previously, the observed differences in plasma Lp[a] levels cannot be attributed solely to differences in apo[a] allele size distributions. For example, the African-Americans and Caucasians had very similar distributions of apo[a] allele sizes and yet strikingly different distributions of plasma Lp[a] levels. In all three populations, the distribution of apo[a] alleles that produced little or no detectable apo[a] protein, as assessed by immunoblotting, tended to mirror the distribution of expressing alleles. Therefore, there is no common null allele in any population.

Dr. Ann White described studies she performed in primary cultures of baboon hepatocytes to examine the biosynthesis of Lp[a] and to identify the factors responsible for the observed wide variation in plasma level of Lp[a] production. After steady-state labeling of hepatocytes, apoB co-immunoprecipitated with apo[a] from the culture medium, but not from the cell lysate. A large proportion of apo[a] in the culture medium was in a free form. Apo[a] that was associated with apoB was found in the VLDL density range. Addition of serum to the cultures resulted in recovery of apo[a] in the Lp[a] density range and the virtual disappearance of free apo[a]. These experiments suggest that the assembly of the Lp[a] particle occurs after secretion.

Pulse-chase studies and endoglycosidase digests of intracellular apo[a] demonstrated that apo[a] was synthesized as a lower molecular weight precursor which had a prolonged residence time in the endoplasmic reticulum (ER) before maturation and secretion. Variation in the kinetics of apo[a] maturation was observed between allelic variants with larger isoforms requiring a significantly longer period of time to exit the ER than small isoforms. This may account for the inverse correlation observed between apo[a] size and plasma Lp[a] concentration. In addition, examination of hepatocytes expressing a "null" apo[a] allele that gave rise to an apo[a] transcript in the liver but no detectable plasma protein, revealed the presence of an intracellular apo[a] protein that was retained in the ER and was not secreted. Analysis of hepatocytes from additional animals revealed that "null" apo[a] proteins of different sizes, which are defective for secretion, exist within the population. Efficiency of post-translational processing of apo[a] is thus a major determinant of plasma Lp[a] concentration.

Dr. Hans Joachim Mueller discussed in vitro studies that examined the expression of various human apo[a] cDNA constructs in HepG2 cells. Stably transfected HepG2 lines produced r-Lp[a] particles that migrated to a position between LDL and HDL after density gradient centrifugation. More than 90% of r-apo[a] in the medium of transfected cells was found to be lipoprotein-associated. To determine whether the single unpaired cysteine residue in kringle 36, Cys-4057, played a role in the association of apo[a] with the apoB-100 of LDL, the residue was mutated to either glycine or serine. Expression of both these mutants in HepG2 cells gave rise to free apo[a] glycoprotein that was not associated with lipoproteins. This strongly suggests that there is a disulfide bridge between Cys-4057 of apo[a] and a cysteine residue in apoB-100.

Structural domains that mediate the lysine-binding properties of apo[a]/Lp[a] were identified by introducing both point mutations and deletions into a recombinant apo[a] construct that contained 18 kringle 4 repeats. The characterization of mutant r-apo[a]/Lp[a] by lysine-Sepharose affinity chromatography revealed three lysine-binding sites (LBS) within apo[a]. LBS I was localized to kringle 37 and accounts for the lysine-binding properties of r-Lp[a] particles. The domains comprising LBS II+III are masked within r-Lp[a] particles and become exposed in the presence of 1% Tween.

A two-step model was presented for the assembly of the apo[a]/apoB-complex in Lp[a]: positioning of Cys-4057 in apo[a] and its counterpart in apoB might be achieved by interactions of LBS II in apo[a] and lysine residues in apoB before both proteins become covalently linked in the second step. To test this model, two further deletion mutants lacking either kringles 32–35 or kringle 32 alone were expressed in HepG2 cells. In contrast to wild type apo[a] [> 90% complexed to apoB], about 50% of the mutant Δ32 and more than 90% of the mutant Δ32–35 were found as free apo[a] glycoprotein, indicating that the presence of kringle 36 containing Cys-4057 is not

sufficient for the apo[a]/apoB association and that kringle domains 32–35 contribute to the Lp[a] assembly, probably by LBS II which is localized in this region.

Finally, **Dr. Roberto Taramelli** reviewed his work on the characterization of the apo[a] and plasminogen gene complex on chromosome 6. He has successfully cloned and characterized the entire apo[a] and plasminogen genes using yeast artificial chromosomes. The apo[a] gene and plasminogen genes are within 50 kb of each other and are oriented in a head-to-head fashion. Interestingly, there is another apo[a]-like gene in the cluster which, like the apo[a] gene, contains a signal sequence and kringle-like repeats. This gene is expressed in the liver. Efforts are now being made to characterize this gene.

To determine whether differences in the sequence of the 5' flanking region contributed to the observed differences in plasma levels of Lp[a], the effect of a 1.4 kb fragment was analyzed with a luciferase assay after transfection into HepG2 cells. This region was shown to be responsible only for basal activity of the gene. Additional sequences, located either upstream or in the large intron between the first and second exon, are being screened for the presence of microheterogeneity that could be related to different Lp[a] levels.