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## Meeting Summary

# The 18th Annual Meeting of the European Lipoprotein Club

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The European Lipoprotein Club met September 11th to the 14th, 1995, in Tutzing, Germany. There were 101 participants from 12 European countries and the United States. Warren J. Strittmatter (Durham, NC) opened the meeting with a state-of-the-art lecture on "Apolipoprotein E and Alzheimer's Disease." Inheritance of specific apoE alleles in large part determines the risk and mean age of onset of late-onset and sporadic AD. Patients with late-onset AD have a higher probability of having the *APOE4* allele (50% at age 75 years), whereas the *APOE2* allele is associated with a later age of onset. Dr Strittmatter discussed the possible mechanisms whereby apoE isoforms contribute differently to disease expression. In his opinion, identifying the isoform-specific pathways of apoE metabolism is central to the development and testing of hypotheses on the pathogenesis of AD.

Isoform-specific differences have been identified in the binding of apoE to the MAP- $\tau$ , which forms paired helical filaments and neurofibrillary tangles, and to  $\beta$ -amyloid peptide, a major component of the neuritic plaque. An unresolved issue of great importance is the relationship between structural pathological lesions and the cellular pathogenesis responsible for the major signs and symptoms of disease, namely, progressive dementia. Identification of apoE in the cytoplasm of human neurons and characterization of isoform-specific binding of apoE to the MAP- $\tau$  and MAP-2 present the possibility that apoE may affect microtubule function in the brain of AD patients. Dr Strittmatter concluded his presentation with a report on studies that have found differences in the cross-linking of apoE isoforms to lipid peroxidation products in vitro (apoE3 > apoE4); in cerebrospinal fluid these products also cross-link with apoE. In the lively discussion that followed, Dr Strittmatter defined the use of apoE genotyping in AD (1) as a diagnostic tool to confirm AD and (2) for risk analysis in a person without clinically evident AD.

The first session on "Novel Functions of Apolipoproteins and Lipoprotein Receptors" began with an excellent talk by Wolfgang J. Schneider (Vienna, Austria), during which he summarized new findings from his laboratory on the molecular genetics of lipoprotein receptor expression in the laying hen model. These findings place the *LDLR* gene family at the crossroads of lipoprotein me-

tabolism and cell differentiation. Deposition of yolk lipoproteins into chicken oocytes is mediated by a receptor that belongs to the *VLDLR* subgroup of the *LDLR* supergene family (Bujo et al. *EMBO J.* 1994;13:5165-5175.). Recent additional characterization of the details of this receptor, termed OVR, has revealed other surprising features. First, a splice-variant form that contains an additional 90-nucleotide exon corresponding to an *O*-glycosylated region usually found in *LDLRs* has been identified. Interestingly, the longer transcript and protein are present at the highest levels in exactly those tissues that express *VLDLRs* in mammals, but the smaller protein is found almost exclusively in the gonads, ie, the ovary and testes. Second, Schneider and colleagues have demonstrated that OVR binds and internalizes  $\alpha$ 2M, hitherto known as a ligand of LRP only. Since  $\alpha$ 2M not only inactivates proteinases but also carries and/or modulates certain growth factors and cytokines, it appears that both types of germ cell utilize the smaller form of the receptor to obtain, in addition to lipoproteins, nonlipoprotein components important for growth and differentiation. Thus, the evidence strongly suggests that OVR is involved in maturation of female and male germ cells. Third, the mutation in OVR that causes atherosclerosis and female sterility in an animal model of familial hypercholesterolemia, the "restricted ovulator" hen, has been determined. This is the first naturally occurring mutation in a receptor of the *VLDLR* group. Again, the failure of this mutant to reproduce links abnormal gonadal development to lipoprotein metabolism and places OVR at a crucial position in the paths of lipoprotein metabolism and regulation of cell differentiation.

Roméo Cecchelli (Lille, France) reported that transcytosis of LDL across the blood-brain barrier appears to be a "new" function of the *LDLR*. In a coculture of astrocytes and brain capillary endothelial cells on opposite sides of a filter, evidence was found for a soluble factor that regulates LDL transcytosis, which appears to be driven by the cholesterol content of the astrocytes. Blocking this *LDLR* with the monoclonal antibody C7, which is known to interact specifically with the receptor-binding domain, abolished transcytosis of LDL. Radiolabeled LDL was not degraded during transcytosis, thereby indicating that this "new" pathway is different from the classic *LDLR* pathway whereby LDL is directed to the lysosomes for degradation. The use of filipin, a drug that disrupts caveolar trafficking, blocked LDL transcytosis to the endothelial cells, thus indicating involvement of the caveolae in the *LDLR* transcytosis pathway.

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(*Arterioscler Thromb Vasc Biol.* 1996;16:898-904.)

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## Selected Abbreviations and Acronyms

A-I <sub>Milano</sub> /A-II/A-I-KO	= apoA-I <sub>Milano</sub> /apoA-II/apoA-I knockout mice
$\alpha$ 2M	= $\alpha$ 2-macroglobulin
AD	= Alzheimer's disease
C/EBP- $\alpha$	= CCAAT/enhancer binding protein- $\alpha$
FH	= familial hypercholesterolemia
GPDH	= glyceraldehyde 3-phosphate dehydrogenase
HL	= hepatic lipase
HNF3	= hepatocyte nuclear factor-3
LCAT	= lecithin-cholesterol acyltransferase
LPL	= lipoprotein lipase
LPS	= lipopolysaccharide
LRP	= LDL receptor-related protein
MAP	= microtubule-associated protein
MNP-3	= mononuclear phagocyte-3
NAME	= nitroarginine methyl ester
NMMA	= N <sup>G</sup> -monomethylarginine
OVR	= oocyte vitellogenesis receptor
PAF(AH)	= platelet-activating factor (acetylhydrolase)
RHDL	= reconstituted HDL
SAA	= serum amyloid A
(V)LDLR(s)	= (V)LDL receptor(s)
WHHL	= Watanabe heritable hyperlipidemic
YAC	= yeast artificial chromosome

Richard James (Geneva, Switzerland) presented data on injury-induced expression and release of apoE and apoJ (clusterin) from cultures of embryonic rat spinal cord neuronal cells. Within 24 hours of induction of lethal stress by glutamate treatment (specific for neurons), there was an increase in apoE and apoJ mRNA content and release of these apoproteins, both of which events could be blocked by the N-methyl D-aspartate receptor antagonist MK-801. Both apoproteins were associated immunohistochemically with neuronal and nonneuronal cells, and expression of apoE and apoJ in both cell types was also induced by nonspecific initiation of cell death. Given that both proteins are associated with the plaques of AD, these data may be relevant to the pathogenesis of this disease.

Winfried März (Freiburg, Germany) presented the results of a study of 200 histomorphologically staged AD brain samples, in which the  $\epsilon$ 4 allele was correlated with the amount of amyloid in the brain and the formation of neurofibrillary tangles. In vitro studies of  $\beta$ -VLDL- $\beta$ -amyloid 4 complexes, in which  $\beta$ -amyloid 4 is associated with the apoE moiety of  $\beta$ -VLDL, showed increases in uptake of the complexes, which subsequently were not degraded. The evidence provided suggests that LRP could be the receptor responsible for uptake of these complexes and that  $\beta$ -amyloid 4 is the peptide that modifies intracellular trafficking of internalized ligands.

Markus Haumer (Vienna, Austria) reported on the costaining of lactoferrin and apo E in brain slices from autopsied patients with AD. Lactoferrin, a known ligand of the LRP, was recovered from the bloodstream into endosomes in an SDS-stable complex with LRP. Participation of lactoferrin under such conditions and at sites of inflammation was assumed from postmortem brain tissue slices of patients with multiple sclerosis. At acute inflammatory foci, apoE-lactoferrin costaining was dramatically enhanced, preferentially within macrophages that had in-

filtrated activated glial cells. The cell-surface staining of macrophages disappeared at late-focus inflammatory sites with lactoferrin but not at those with apoE; lactoferrin stained at a single intracellular region, whereas apoE showed a staining pattern that resembled ongoing endocytosis. The interplay of apoE and lactoferrin, both ligands for the LRP, is being investigated further in tissue cultures of glia-supported neurons.

Armin Steinmetz (Vienna, Austria) gave an overview of the lipoprotein and nonlipoprotein functions of human apoA-IV. He presented new data that emphasized the unusual appearance of this hydrophilic plasma apoprotein, which is predominantly associated with pre- $\beta$ -migrating lipoprotein particles, and its role in reverse cholesterol transport. ApoA-IV/phosphatidylcholine complexes and apoA-IV-containing particles isolated from human plasma are capable of promoting cellular cholesterol efflux; pre- $\beta$ -migrating apoA-IV particles can serve as primary acceptors of cell-derived cholesterol, as shown by two-dimensional gel electrophoresis. Free (unbound) cell-derived cholesterol can be esterified by the enzyme LCAT in the presence of apoA-IV as a cofactor. By using deletion mutants of the protein that are expressed in *Escherichia coli*, the relevant domain in apoA-IV for cofactor activity can be localized between amino acids 117 and 160. A novel function of apoA-IV that is not shared by apoA-I and appears distinct from other functions described so far was reported by P. Tso and colleagues, namely, suppression of food intake in the rat by apoA-IV when infused intravenously or given intracerebroventricularly (*J Clin Invest.* 1993;91:1830-1833.). ApoA-IV has also been shown to decrease gastric acid secretion when given intracerebroventricularly. In a collaborative study with human apoA-IV, Tso and colleagues also observed suppression of food intake in rats. Subsequent studies of apoA-IV in human cerebrospinal fluid revealed migration of apoA-IV in the pre- $\beta$  position and with the apparent same molecular mass and identical isoelectric focusing patterns as those of apoA-IV in plasma. Further evaluation of this intriguing new function should concentrate on the processes by which apoA-IV reaches the structures of the central nervous system.

Richard Bruckdorfer (London, England) reported the inhibitory effects of LDL and HDL on thromboplastin activity that were independent of those associated with tissue factor pathway inhibitor, a protein that, to a large extent, is bound to these lipoproteins. In the case of LDL, this inhibitory activity was found to reside in apoB-100, although oxidation of the lipoprotein reversed this inhibition and ultimately led to procoagulant activity exhibited by LDL. The spectroscopic evidence presented showed the presence of both hydrophobic and ionic interactions between apoB-100 and thromboplastin.

The following talk, by David Riddell (London, England), considered the influence of apoE on NO production in platelets. ApoE-dimyristoylphosphatidylcholine complexes induced potent, dose-dependent inhibition of ADP-induced platelet aggregation in platelet-rich plasma as well as washed platelets. This inhibition was almost completely abolished when the platelets were preincubated with the NO synthase inhibitors L-NMMA and L-NAME (300  $\mu$ mol/L) but not D-NMMA. Other experiments supported the notion that apoE stimulates NO production: its physiological target guanylate cyclase was activated because cAMP levels rose. This is the first re-



port to show that apoE influences NO production, although it remains to be established whether this newly identified link between apoE and NO is widespread.

The session continued with a comprehensive overview of the acute-phase protein SAA by Patricia Woo (London, England). This protein is secreted mainly by hepatocytes and its concentration can increase as much as 1000-fold during inflammation. In the circulation SAA is associated primarily with HDL. The SAA superfamily comprises two acute-phase genes, *SAA1* and *SAA2*; a pseudogene, *SAA3*; and a constitutive 11p15. Both *SAA1* and *SAA2* are found in human amyloid deposits, whereas only *SAA2* is found in amyloid deposits in BALB/c mice. With the use of recombinant SAA and transfectant mutants (Asp8 and 1-11 $\Delta$ ), it has been demonstrated that the N-terminal region of SAA is important for HDL binding. This region also appears to be important for the incorporation of SAA into amyloid deposits, because in the CE/J mouse strain, which is resistant to amyloidogenesis, the major SAA isoform shows mutations in the N-terminal peptide. Furthermore, in a formation assay in vitro, the 1-11 $\Delta$  transfection mutant was unable to form fibrils. From the work of several groups it is apparent that SAA functions may be important in atherosclerosis as well. Recombinant SAA has been shown to have high affinity for cholesterol, and SAA can enhance cholesterol efflux from HDL as well as stimulate uptake of radiolabeled cholesterol from the medium by HepG2 cells. Another property of recombinant SAA is its ability to inhibit thrombin-induced platelet aggregation. SAA mRNA has also been detected in endothelial cells, some smooth muscle cells, and macrophage-derived foam cells from human atherosclerotic lesions.

In the next lecture Daniel M. Levine (New York, NY) discussed the ability of RHDL to neutralize endotoxin. The lipid A domain of LPS not only anchors it to Gram-negative bacteria but also inserts it into HDL. In a mouse lethality model it was shown that intravenous infusion of RHDL, which causes a twofold elevation in circulating HDL levels, led to a fourfold increase in survival compared with control mice that received an intravenous infusion of saline only. Introduction of human *APOA1* into transgenic mice also caused a twofold elevation in HDL levels and a concomitant fourfold increase in survival. Furthermore, in high-HDL mice, tumor necrosis factor- $\alpha$  release was significantly lower than in low-HDL mice. The ability of RHDL, a lipid emulsion (Intralipid) with naturally occurring lipoproteins, to neutralize LPS was tested in an in vitro human whole-blood model of LPS binding protein and CD14-dependent activation of cytokine production. Only phospholipid content was found to be correlated with the effectiveness of LPS neutralization. These data support the hypothesis that LPS binding to phospholipid on the surface of RHDL prevents CD14-dependent activation of tumor necrosis factor- $\alpha$ .

Wie Liao (Huddinge, Sweden) investigated whether endotoxin could alter LDLR expression in rats. Eighteen hours after injection of 500  $\mu$ g endotoxin per 100 g body weight IP, LDLR expression had been reduced by 70%. In contrast, LDLR mRNA levels were markedly higher at this time point. Time-course experiments showed that LDLR expression progressively decreased and that mRNA levels decreased by 78% at 4 hours, returned to baseline levels at 8 hours, and increased by  $\approx$ 60% after 18 hours.

Human serum paraoxonase is an enzyme that is associated with a specific HDL particle that contains apoA-I and apoJ. Michael Mackness (Manchester, England) reported that purified paraoxonase is capable of preventing the accumulation of lipid peroxides in LDL that has been incubated under oxidizing conditions. PAFAH is active on both HDL and LDL and is also capable of hydrolyzing oxidized phospholipids. Paraoxonase was purified to homogeneity from human HDL and its ability to hydrolyze PAF was investigated. Although paraoxonase was able to hydrolyze PAF at a rate of 10 nmol  $\cdot$  min $^{-1}$   $\cdot$  kg $^{-1}$  with a  $K_m$  of 10.1  $\mu$ mol/L, its activity was  $\approx$ 1000-fold lower than that of LDL-associated PAFAH. Mackness and colleagues have postulated that both PAFAH and paraoxonase may act in concert to hydrolyze lipid peroxides that are generated on LDL.

Ulrike Beisiegel (Hamburg, Germany) described the identification of a putative new apolipoprotein of 110 kDa that was initially detected during catabolic studies with  $^{125}$ I-labeled chylomicrons. Digestion of the protein with trypsin and V8 yielded four peptides that have  $>80\%$  homology with human complement factor H-related proteins. After polyclonal antibodies had been generated against the purified protein, it was also detected in HDL. Incubation of human plasma with Intralipid and separation of the TG-rich lipoproteins by ultracentrifugation led to enrichment of this fraction with the 110-kDa protein. A HepG2 cDNA library is presently being screened with the polyclonal antibody to clone the gene for the 110-kDa protein.

The final talk of this session was given by Gregor Roth (Regensburg, Germany), during which he described characterization of circulating blood monocyte subpopulations by flow cytometry. Five subpopulations were identified on the basis of differential expression of CD14, CD64, CD16, CD32, CD33, and CD56. The distribution of these monocyte subpopulations was studied in healthy probands ( $n=55$ ) and patients with FH ( $n=19$ ). HDL cholesterol levels were negatively correlated with the size of the MNP-3 population in FH patients, and apo E3/3 and E4/4 phenotypes were associated with an increase in the MNP-3 population in healthy probands and FH patients. Expression of the CD45RA activation antigen by blood MNPs showed a positive correlation with plasma levels of LDL and Lp(a). Dr Roth suggested that systemic abnormalities in MNPs are related to the chronic inflammatory process within atherosclerotic lesions of the vessel wall.

The second session of the meeting, "Innovative Animal Models for Lipoprotein Research: Transgenics, Knockouts Towards Gene Therapy," began with an excellent lecture by Edward Rubin (Berkeley, Calif) about Lp(a) and its role in atherosclerosis. Lp(a) is present in humans, monkeys, and surprisingly the hedgehog but is absent in other species, which implies that the most frequently used laboratory animals, ie, mice, rats, and rabbits, do not naturally have it. Use of DNA technology has made it possible to create animal strains that express apo(a). Transgenic mice that express human apo(a) have been bred, and expression of the gene is controlled by the promoter of another mouse gene. However, this method has created a problem for study of the regulation of apo(a) gene expression. Dr Rubin presented information on a YAC construct composed of 275 kb of human DNA that contains the entire apo(a) gene and  $>60$  kb of the 5' and



3' noncoding regions. After injection of this construct into fertilized mouse oocytes, four lines were obtained in which no rearrangement or fragmentation of the huge DNA fragment had occurred. Plasma levels of human apo(a) in these mice ranged from 8 to 40 mg/dL. The apo(a) mRNA was expressed only in the liver, indicating that the DNA construct contained all or most of its regulatory regions, at least with regard to tissue specificity.

Apo(a) has also been claimed to be an acute-phase protein. In Dr Rubin's experiments an acute-phase reaction in apo(a)-transgenic mice was induced by injection of turpentine. As an indicator for the acute-phase reaction, haptoglobin mRNA levels were found to have increased fivefold. However, there was no increase in the amount of apo(a) mRNA, indicating that apo(a) is not an acute-phase protein.

Apo(a) has also been claimed to be under hormonal control. Apo(a) mRNA levels were measured before and after orchietomy in apo(a)-transgenic mice, and an approximate twofold to threefold increase in the amount of mRNA was observed after the procedure. This finding confirms that male hormones control expression of apo(a). Upregulation of apo(a) mRNA could be abolished by treating the animals with either testosterone or estrogen, and it was surprising that both hormones had a similar effect on apo(a) expression.

Transgenic mice that express human apo(a) do not have Lp(a), because mouse LDL does not bind to apo(a). To construct Lp(a)-bearing mice, transgenic mice that expressed human apoB were made and then mated with transgenic mice that expressed human apo(a). Because only low-expressor apoB-transgenic mice were used, most of the human apoB was bound to human apo(a). The LDL-VLDL ratio was calculated in control, low-expressor human apoB-transgenic, human apo(a)-transgenic, and Lp(a)-bearing mice. Relative to controls, the ratio was only slightly elevated in apoB-transgenic and Lp(a)-bearing mice and somewhat more elevated in apo(a)-transgenic mice. However, when atherosclerotic lesions were examined, apoB-transgenic mice had only slightly more lesions than did control mice, but human apo(a)-transgenic mice had an  $\approx 10$ -fold increase in lesions and those mice that expressed both human apo B and apo(a) had an  $\approx 20$ -fold elevation in lesion area compared with controls. These findings indicate that free apo(a) per se is an atherogenic protein and that apo(a) bound to LDL is even more atherogenic.

Dr Rubin also presented data on apo(a)-human apoB interactions, which are known to be due to a disulfide bond. In apo(a) the Cys at amino acid 4057 is known to be involved in disulfide bonding, but in apoB there are four Cys's at the carboxy-terminal end (at amino acids 3734, 3890, 4190, and 4326) that are potential sites for disulfide bonding. Dr Rubin showed that when the Cys at position 4326 was altered, all apo(a) was lipid-free, indicating that this Cys is the one responsible for the disulfide bond between apo(a) and apoB.

In the final part of his lecture, Dr Rubin discussed the consequences of enhanced apoB editing on Lp(a)-bearing mice. Protein p27 has been shown to cause the C $\rightarrow$ U change in apoB mRNA that results in the manufacture of apoB-48 instead of apoB-100. An adenovirus construct that contained the p27 gene was injected into rabbits. These animals do not normally edit apoB, but after injection with the p27 construct, the rabbits were found to have

apo B-48 in their plasma. When the same construct was injected into Lp(a)-transgenic mice, plasma levels of Lp(a) were found to be only one fifth of their original values. This means that by altering apoB editing, it is possible to lower the amount of apoB-100-containing LDL in plasma, which would in turn make the lipoprotein profile less atherogenic.

The next speaker, Francisco Blanco-Vaca (Barcelona, Spain), presented his work on human apoA-II in three transgenic mouse lines with plasma concentrations of apoA-II ranging from 21.4 to 74.4 mg/dL. In these mice, human apoA-II was expressed exclusively in the liver and the protein was found only in HDL particles. When mice of the highest-expressor line were fed a standard chow diet, mRNA levels for mouse apoA-I and A-II decreased. However, when these mice were kept on a high-fat diet, no decrease in mRNA levels was observed. Serum total cholesterol levels decreased and TG levels increased when the mice were fed the chow diet. Lipoprotein analysis revealed that the increase in TGs was due to an increase in VLDL TGs and that the decrease in total cholesterol was due to a decrease in HDL cholesterol. When the mice were kept on the high-fat diet, HDL cholesterol levels in control mice decreased, but those in high-expressor apoA-II-transgenic mice increased. Potential mechanisms for this paradoxical rise were discussed.

Giulia Chiesa (Milan, Italy) presented studies on transgenic mice that express the human variant apoA-I<sub>Milano</sub>, which is characterized by a Cys $\rightarrow$ Arg substitution at position 173. Carriers of this apoA-I variant have dramatically lower HDL cholesterol levels but an apparent surprising absence of cardiovascular disease. ApoA-I<sub>Milano</sub> is known to exist as a homodimer as well as a heterodimer with human apoA-II. Since mouse apoA-II does not form a heterodimer with apoA-I<sub>Milano</sub>, mice that express both human apoA-I<sub>Milano</sub> and human apoA-II were created. To better evaluate the effects of apoA-I<sub>Milano</sub>, these double-transgenic mice were then mated with apoA-I-knockout mice, resulting in A-I<sub>Milano</sub>/A-II/AI-KO. Mice that expressed human apoA-I and A-II on an apoA-I knockout background were used as controls. The A-I<sub>Milano</sub>/A-II/AI-KO had higher serum TG levels and lower HDL cholesterol levels. HDL was present in two subclasses, as it is in humans, and the size and density of HDL particles resembled that of human HDL. In these A-I<sub>Milano</sub>/A-II/AI-KO, apoA-I<sub>Milano</sub> formed homodimers and heterodimers as it does in human plasma. With this new mouse line the effects of apo A-I<sub>Milano</sub> can be studied in greater detail.

Anja Mehlum (Oslo, Norway) presented a study on human LCAT-transgenic mice. A high-expressor human LCAT-transgenic mouse line was created, and LCAT activity in these mice was  $\approx 40$ -fold higher than normal. This overexpression of LCAT resulted in decreased serum TG levels. Although LPL activity remained unchanged, HL activity increased markedly. LDL and VLDL cholesterol levels decreased while HDL cholesterol increased by  $\approx 20\%$  and the size of HDL particles also increased by  $\approx 20\%$ . Cholesterol efflux-stimulating activity of HDL from these transgenic mice was nearly doubled when compared with controls. Most likely, these LCAT-transgenic mice have a less atherogenic lipoprotein profile than do control mice.

Omar Francone (Groton, Conn) discussed studies of transgenic mice that express a moderate increase in LCAT activity (1.2- to 1.6-fold of the normal value). A



20% to 60% increase in HDL cholesterol was also observed. These LCAT-transgenic mice were mated with human apoA-I- and/or human apoA-II-transgenic mice, thereby producing three mouse lines. HDL cholesterol levels were twofold higher and the HDL particle size was larger in apoA-I/LCAT- and apoA-I/apoA-II/LCAT-transgenic mice compared with apoA-I-, apoA-II/LCAT-, and LCAT-transgenic mice.

Catherine Fievet (Lille, France) presented a study of transgenic rabbits. Six transgenic lines were obtained after injection of a 12.5-kb DNA fragment (containing the entire human apoA-I gene) into oocytes from New Zealand White rabbits. The level of human apoA-I was 8 to 100 mg/dL and the level of rabbit apoA-I was reduced. In the highest-expressor line, HDL cholesterol levels increased from 18 to 49 mg/dL and total cholesterol from 46 to 76 mg/dL. HDL appeared in only one size fraction, as in control rabbits, and not in two size fractions, as in humans and transgenic mice that express human apoA-I. There was a linear correlation between plasma human apoA-I and HDL cholesterol levels. These transgenic rabbits were then mated with the LDLR-deficient WHHL rabbit. Levels of human apoA-I were  $\approx$ 100 mg/dL in these offspring and their HDL levels were significantly higher than in the LDLR-deficient rabbits. The non-HDL cholesterol to HDL cholesterol ratio was fourfold lower in WHHL rabbits carrying the human apoA-I gene compared with control WHHL rabbits. In WHHL rabbits expression of human apoA-I did not decrease the expression of rabbit apoA-I.

Marten Hofker (Leiden, Netherlands) presented studies about mice deficient in both apoE and apoC-I. The genes for these proteins are located in a conserved cluster in both humans and mice. Mice that lack both genes cannot be obtained by mating because of the proximity of the genes. This problem was overcome by consecutive targeting. First, the apoE gene was replaced by that for neomycin resistance; then these embryonic stem cells (lacking apoE) were targeted with a construct in which the apoC-I gene had been disrupted with a hygromycin gene. ApoE, C-I, and C-II probably share common regulatory regions. In mice that lacked both apoE and apoC-I, expression of apoC-II was also affected and was only  $\approx$ 60% of normal. Mice that lack apoE only have  $\approx$ 68% of normal apoC-I mRNA levels, and in those that lack apoC-I the amount of apoE mRNA is decreased by 50%.

Francesco Acquiati (Mannheim, Germany) presented work about apo(a) expression. To obtain the very large apo(a) gene and its flanking regions, YAC clones had to be used. Because different apo(a) alleles have very different expression activities and because high levels of apo(a) expression were needed, a YAC library was constructed with material from a subject who had a high plasma level of apo(a); this allele was subsequently cloned. This 370-kb fragment contained the entire apo(a) gene and 40 kb of the 5' and 150 kb of the 3' flanking sequences. When this construct was microinjected into mouse oocytes, one transgenic line expressing human apo(a) was obtained. The integrated DNA construct did not contain any structural rearrangements.

The second half of this session began with a presentation by Seppo Ylä-Herttuala (Kuopio, Finland). He gave an excellent review about the techniques that are used in gene therapy and the problems that are associated with these techniques. Retroviruses, the most widely used

vectors in gene therapy, are RNA viruses in which structural genes between two long tandem repeats can be replaced by the gene of interest. Retroviruses become integrated with chromosomal DNA and therefore, their expression is stable. However, mitosis is required for retroviral material to be incorporated, which implies that there is a potential danger for insertional mutagenesis, although so far no reports of this have appeared. Another problem with retroviruses is size restraint, since only 7 to 8 kb can be inserted into a retrovirus construct.

Adenoviruses have great potential for use in gene therapy. They infect a wide variety of cells and no mitosis is needed for incorporation of adenoviral material. One limitation is that they do not become integrated with the genomic DNA, and thus their expression is only transient. On the other hand, because there is no integration, there is no danger of insertional mutagenesis. Adenoviruses may cause problems with the immune response because repeated adenovirus infections will have no effect. At present third-generation adenovirus vectors are available, but these vectors cannot yet be used in the treatment of chronic diseases. Liposomes are safe vectors, since they do not contain any viral DNA. They do not integrate and thus, there is no danger of insertional mutagenesis. Because there is no integration their expression is only transient, and the transfection efficacy of liposomes is poor. These liposomal vectors could be used in situations in which transient expression or inefficient transfection is sufficient, such as for treatment of restenosis after angioplasty.

Wolfgang Hofmann (Graz, Austria) discussed transgenic mice that express human LPL. LPL is produced primarily in cardiac and skeletal muscle and adipose tissue. Tissue-specific expression of LPL was studied with a DNA construct that contained either 4.6 or 8.0 kb of the upstream region of the mouse LPL gene linked to a human LPL minigene. Mouse lines in which the 4.6-kb construct was integrated did not express human LPL at all. Lines that contained the 8.0-kb construct had a high level of human LPL expression in cardiac muscle only. When these transgenic mice were mated with LPL knockout mice, TG levels in their offspring were dramatically reduced; mice homozygous for the LPL deficiency and without human LPL died during the first day of life, whereas mice homozygous for the mouse LPL deficiency but with human LPL expression in cardiac muscle survived. These transgenic mice demonstrate that it is sufficient to have LPL expression in only one tissue to control plasma TG levels.

Peter Weinstock (New York, NY) also presented a study on LPL knockout mice, in which the endogenous LPL gene had been replaced by a neomycin resistance gene. At birth homozygotes for the LPL deficiency had threefold and sevenfold elevations in TG and VLDL cholesterol levels, respectively. When allowed to suckle these mice died 18 hours after birth. At that time their TG levels were  $\approx$ 15 000 mg/dL (188 mg/dL in controls). The reason for premature death in these mouse pups is most likely chylomicron inhibition of contact between red blood cells and the endothelium in lung capillaries, thus preventing normal gas exchange. Heterozygotes for LPL deficiency survived to adulthood but had mild hypertriglyceridemia. VLDL turnover studies revealed a decrease in VLDL clearance but no increase in VLDL production.



Thus, in mice a complete LPL deficiency is lethal and a partial LPL deficiency has physiological consequences.

The last talk in this session was given by Jesus Osada (Zaragoza, Spain) on HL knockout mice. Mice homozygous for this deficiency had mild hypercholesterolemia, but their TG levels were not altered. On a high-fat diet the HDL cholesterol level was about doubled in HL knockout mice, whereas it was slightly less than normal in controls. These findings demonstrate that HL has an important role in HDL metabolism.

The third session on "Fatty Acid Metabolism: Regulation and Clinical Implications" began with a comprehensive overview by Peter Arner (Huddinge, Sweden) on adipocyte lipolysis. It is well established that adipose tissue is a heterogeneous metabolic organ. Lipid mobilization via lipolysis in fat cells is more pronounced in visceral fat depots than in peripheral subcutaneous regions. This difference can be attributed to regional changes in the action of the major lipolysis-regulating hormones, catecholamines and insulin. Catecholamines are more and insulin is less lipolytic in visceral compared with subcutaneous fat cells. The catecholamine difference is attributed to changes in the expression and function of adrenergic receptors. The lipolytic  $\beta_1$ ,  $\beta_2$ , and  $\beta_3$ -adrenergic receptors are more prominent in visceral fat cells, whereas the antilipolytic actions of  $\alpha_2$ -adrenergic receptors are more pronounced in subcutaneous cells. Insulin is less lipolytic in visceral fat cells because of a combination of decreased receptor affinity and decreased signaling through the phosphatidylinositol kinase 3 pathway.

Dr Arner went on to discuss the metabolic (insulin resistance) syndrome, in which elevated levels of free fatty acids are commonly observed. This may be partially due to a further increase in the regional differences in lipolysis. Catecholamine action is inhibited in subcutaneous fat cells owing to a decrease in the expression and function of the  $\beta_2$ -adrenergic receptor plus a decrease in the final rate-limiting step of lipolysis activation by hormone-sensitive lipase. However, catecholamine action on lipolysis is increased in visceral fat cells owing to an increase in function of the  $\beta_3$ -adrenergic receptors combined with a decrease in function of the  $\alpha_2$ -adrenergic receptors. These concurrent processes enhance lipid mobilization from visceral fat cells and decrease lipid mobilization from subcutaneous fat cells after catecholamine stimulation in the metabolic syndrome. Thus, portal levels of free fatty acids will be markedly elevated, and these in turn can cause a number of the metabolic changes that are typically observed in the syndrome, such as hyperinsulinemia, increased VLDL TG production, and increased glyconeogenesis.

Finally, Dr Arner discussed another condition in which alterations in adipocyte lipolysis may be important, namely, familial combined hyperlipidemia. He pointed out that this condition is associated with lipolytic catecholamine resistance in subcutaneous fat cells owing to a selective defect in expression of hormone-sensitive lipase. In addition, the ability of fat cells to synthesize TGs is impaired. The combination of these two defects causes a decrease in TG turnover in fat cells, which in turn may lead to a situation in which adipose tissue is "uncoupled" from the route whereby free fatty acids in VLDL are stored in and then released from fat to be taken up by the liver for synthesis of new VLDL TGs. If adi-

pose tissue is "uncoupled" from normal VLDL metabolism, then the capacity of other pathways to accommodate the increased VLDL "traffic" may be insufficient, so that VLDL may accumulate in the blood and cause hypertriglyceridemia.

Gunnar Bjursell (Göteborg, Sweden) described the molecular mechanisms of adipocyte differentiation. Mature adipocytes arise from multipotent stem cells. After determination adipoblasts are formed, which then develop into preadipocytes that express early markers of the adipocyte phenotype. Subsequent differentiation leads to establishment of adipocytes that express late markers for fat cell development. Dramatic changes in cell morphology and gene expression occur during development. Late events in adipogenesis include activation of many adipocyte-specific genes. The transcription factor C/EBP- $\alpha$  has been shown to transactivate a number of such genes. By culturing 3T3-F442A preadipocytes with or without the two adipogenic hormones insulin and hydrocortisone, Dr Bjursell could monitor the gradual activation of C/EBP- $\alpha$  as well as one early marker, LPL, and one late marker, GPDH, for adipocyte differentiation under different conditions. Hydrocortisone treatment prevented development of the adipocyte phenotype as well as expression of GPDH and C/EBP- $\alpha$ . Stably transfected 3T3-F442A preadipocytes with an "antisense" C/EBP- $\alpha$  expression plasmid reduced lipid accumulation after the cells had differentiated into mature adipocytes. Lipid accumulation appeared to be correlated in a linear manner with the level of C/EBP- $\alpha$  protein.

LPL is one of the first markers of adipocyte differentiation. Transient transfection studies by Bjursell's group revealed the existence of several positive and negative elements in the LPL promoter. Possible regulatory elements were also identified in the introns and the 3' flanking sequences by a DNase I hypersensitivity assay. Stable transfection of different 5' deletion mutants in 3T3-F442A preadipocytes was established, and gradual activation of the LPL promoter during preadipocyte differentiation was monitored by chloramphenicol acetyltransferase assays at different times. Two regions, named LP- $\alpha$  and LP- $\beta$ , were found to be necessary for differentiation-linked activation of the LPL promoter. Previously unidentified members of the HNF3/forkhead transcription factor family were shown to interact with motifs in LP- $\alpha$  and LP- $\beta$ . This is the first example of the involvement of HNF3/forkhead members in regulating adipocyte-specific gene expression.

The role of fatty acids as products or regulators of lipoprotein metabolism was discussed further in this session. Long-chain fatty acids can modulate the expression of LPL in adipocytes cultured in vitro by increasing transcription levels while decreasing the levels of activity and secretion (Gérard Ailhaud; Nice, France). This effect was dependent on the type of fatty acid: absent with short- or medium-chain fatty acids, but increasing with the length of long-chain fatty acids. According to Martin Bergö (Umeå, Sweden) LPL synthesis in rat adipose tissue modulates most of the LPL activity at basal levels, whereas during short-term fasting/feeding periods, other mechanisms that completely suppress LPL activity operate at the posttranscriptional level.

In humans levels of fatty acids that are released by adipose cells in subcutaneous veins have been shown to reflect VLDL production and to be correlated with plasma TG and



apoB levels (Simon Coppack; London, England). Moreover, in postprandial periods after sequential meals, nonesterified fatty acids released by LPL from chylomicrons produced after the second meal were found to have originated from TGs produced after the first meal (Keith Frayn; Oxford, England). On the other hand, unsaturated fatty acids were the most potent stimulators of lipoprotein secretion when added to either cultured CaCo cells (Marleen van Greevenbroek; Utrecht, Netherlands) or primary cultured chicken hepatocytes (Philippe Legrand; Rennes, France) compared with saturated fatty acids. Furthermore, increases in  $\Delta 9$ -desaturase activity seemed to correlate with increases in VLDL production in this latter model. Fatty acids also could modulate T-lymphocyte activation in vitro (Thomas Stuling; Vienna, Austria). In humans a DNA variant (Asn<sub>291</sub>→Ser) that alters LPL activity has been found in cohorts of subjects with angiographically assessed coronary artery disease. This variant appeared even more frequently in subjects with the phenotype of familial combined hyperlipidemia (Taco Bruin; Amsterdam, Netherlands).

Philippe Moulin (Lyon, France) presented a case of autoimmune LPL deficiency. In this patient anti-LPL immunoglobulins were detected bound to LPL on the surfaces of circulating chylomicrons.

The 19th Annual Meeting of the European Lipoprotein Club is scheduled for September 9th to 12th, 1996, in

Tutzing, Germany. It will begin with a state-of-the-art lecture on gene therapy, followed by three sessions that will examine (1) the genetics of lipoprotein disorders, (2) lipoproteins and the kidney, and (3) causes and consequences of the metabolic syndrome. For information please contact Prof Guido Franceschini, Secretary, European Lipoprotein Club, Centro E. Grossi Paoletti, Institute of Pharmacological Sciences, Via Balzaretti 9, 20133 Milan, Italy. Fax: 39/2/6470594.

### **The European Lipoprotein Club Organizing Committee**

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