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ELABORATION OF SEPARATION AND INVESTIGATION METHODS OF LIPOPROTEIN MACROMOLECULES FOR RADIOPHARMACEUTICAL APPLICATIONS

Ph. D. Dissertation

ZOLTÁN BOZÓKY

Supervisor: István Antal, Ph.D.

Semmelweis University Department of Pharmaceutics

Consultant: Gyozo Jánoki, Ph.D.

"Fodor József" National Center of Public Health – "FJC" National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary

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ABBREVIATIONS

Apo	apolipoprotein
ACAT	acyl-cholesterol acyltransferase
CAD	coronary artery disease
CHD	coronary heart disease
CM	chylomicron
CETP	cholesteryl ester transfer protein
Da	Dalton
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
FFA	free fatty acid
FC	free cholesterol
HC	hypercholesterolemia
HDL	high-density lipoprotein
HL	hepatic lipase
HLP	hyperlipoproteinemia
HTG	hypertriglyceridemia
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HNE	4-hydroxynonenal
IDL	intermediate-density lipoprotein
LCAT	lecithin-cholesterol acyltransferase
LDL	low density lipoprotein
LPL	lipoprotein lipase
LPO	lipid peroxidation
MDA	malondialdehyde
MMPs	metalloproteinases
NC	normocholesterolemic
Ox-LDL	oxidized low-density lipoprotein
PBS	phosphate buffered saline
PEG	polyethyleneglycol
PL	phospholipids
PLTP	phospholipid transfer protein
PUFA	polyunsaturated fatty acids
RLP	receptor related protein
ROS	reactive oxygen species
SD	standard deviation
S f	Svedberg flotation unit
SMC	smooth muscle cell
SOD	superoxide dismutase
SR	scavenger receptor
TBARS	thiobarbituric acid-reactive substances
TC	total cholesterol
TG	triglyceride
TRL	triglyceride rich lipoprotein
VLDL	very low density lipoprotein
WHHL	watanabe heritable hyperlipidemic rabbits

Ph.D. Thesis

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BY ZOLTÁN BOZÓKY

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1. INTRODUCTION

Atherosclerosis-associated cardiovascular disease is the leading cause of death in most countries. An elevated level of plasma low-density lipoprotein (LDL) cholesterol is an independent risk factor for atherosclerosis. Low density lipoprotein (LDL) is the major transport protein for endogenous cholesterol in human plasma. A great deal of evidence has indicated that oxidative modified LDL plays a critical role in the initiation and progression of atherosclerosis. Atheroscerosis and its main complication infarctions frequently end in sudden death. A key problem of the disease is its silent stepwise progression over time towards its acute manifestation. There is a strong need for non-invasive techniques in directly imaging atherosclerotic lesions for early lesion detections. The available non-invasive imaging techniques such as angiography, ultrasound, and computer tomography are much better at defining the extent of more advanced atherosclerotic lesions than discovering earlier changes of the vascular wall. There has been growing interest in radioisotopic atherosclerotic imaging for early lesion detection, when the lesions are metabolitically most active and therapeutic interventions could even be more beneficial. Low Density Lipoproteins (LDL) are implicated in atherosclerotic process in which blood flow is restricted by cholesterol-related plaque in the vessels and becomes a major component of atherosclerotic plaque lesions. Radiolabelled LDL offer the promising approach to identify the local metabolic fate of these compounds and to study LDL accumulation in vascular tissue, because LDL acts as a trapped ligand in vivo and should be a good tracer for the scintigraphic studies of atheroslerosis. Low-density lipoprotein (LDL) can be radiolabelled with different techniques and various tracers (99mTc, 123I, 125I, 131I, 111In, 67Ga) and it is a tool for the non-invasive exploration of a variety of disorders of lipoprotein metabolism.

We have developed ultra-centrifugation methods to obtain lipoprotein aliquots for radiolabeling and to evaluate lipoprotein labelling techniques, which are suitable for the scintigraphic delineation of experimental atherosclerotic lesions.

Aliquots of sera lipoproteins fractions were taken for Schlieren analysis after adjusting the density of sera and under-layering by salt solution, in the spinning ultracentrifugation capillary band-forming cell. We obtained quantitative results by measuring the Schlieren areas between the sample curves and the reference baseline curve by computerised numerical and graphic techniques. The decomposition of the integrated curve was carried out using a non-linear regression program followed by de-convolution algorithm analysis in order to determine the parameters of the composing Gaussian subclasses. The LDL particle concentrations were calculated from the area under the integral of the Gaussian curve. With this technique, we measured the concentration of LDL and analysed its polydispersity of the aliquots for the preparing of the radioactive labelling lipoproteins. The methods proved to be useful for a clear and immediate visual presentation of the concentration values of lipoproteins in the whole spectra and for the identification of the heterogeneity of lipoproteins subfractions. Combination of preparative and analytical ultra-centrifugation methods allows to investigate lipoprotein aliquots before radioactive labelling with isotopes to identify labelled materials. Density gradient centrifugation allows the simultaneous isolation of the major lipoprotein density classes i.e. VLDL, IDL, LDL, Lp(a), HDL forming discrete bands of lipoproteins in the preparative tubes.

Radiolabelling of LDL with 99mTc was peformed using sodium dithionite as a reducing agent. Radiochemical purity and in vitro stability were controlled by paper chromatography. We have preparated modified lipoproteins with chemical agents and with radioactive irradiations. Labelling efficiency were 85-90 % for human native LDL, for human oxidized LDL, for rabbit native LDL and for rabbit oxidized LDL, for rabbit native IDL and for rabbit oxidized IDL. Rabbits fed a diet containing 1 and 2 % cholesterol for 60 days to develop hyperlipidemia and atheromatous aortic plaques. Autopsies carried out revealed the formation of atheromatous plaques on the inner aortic surfaces of rabbits in induced hyperlipidemia. Gamma scintillation camera scanning of the rabbits was

peformed. Gamma camera in vivo scintigraphy of live rabbits revealed visible signal corresponding to atherosclerotic plaques of aorta and carotid arteries. Our results show that 99m Tc-LDL can be used to assess the organ distribution pattern of LDL in the rabbit, and to detect and localize areas of arterial atherosclerotic lesions. In nude mice developed human tumor cells were detected on the basis of 99mTc labelled LDL with Gamma camera.

Overexpression of receptors for LDL occurs in several cancer cell lines and offers options for drug targeting by using LDL as vechicle. Picture shows that Tc-LDL has the ability to concentrate in the cancer tissue. In vivo scintigraphy revealed visible signal corresponding to tumors in mice and in dogs using radiolabelled lipoproteins. Many carrier systems such as polymers, nanoparticles, microspheres, micelles, as well as liposomes have been applied to prolong the circulation time of certain molecules, to deliver them at the appropriate sites, and to protect them from degradation in the plasma. Liposomes have been widely used as drug carriers. Long-circulating liposomes have been applied for cancer treatment or imaging. Especially, PEG-liposomes have been widely used as a carrier of anti-cancer agents. The lysosomal uptake route, similarly as for LDL. In vitro studies using B16 melanoma cells showed that the liposomes bound exclusively to the LDLr via their apoE moiety than LDL itself. Because of their favorable properties, we anticipate that these apoE-enriched liposomes are advantageous compared with native LDL in the development of a selective LDLr-targeted antitumor materials. It has been well documented that disease states (eg, diabetes, cancer) significantly influence circulating lipoprotein content and composition. It appears possible that changes in the lipoprotein profile would effect not only the ability of the compound to associate with lipoproteins but also the distribution of the compound within the lipoprotein subclasses. Such an effect could alter the pharmacokinetics and pharmacological action of the drug.

2. REVIEW OF THE LITERATURE

2.1. Cholesterol and plasma lipoproteins

Cholesterol is derived to the body from two sources, diet and cellular synthesis. Cholesterol elimination from the body is mediated by the liver, which can transform cholesterol into bile acids. Both free cholesterol and bile acids are secreted into bile (Lin et al.,1980). Dietary and biliary cholesterol is absorbed from the small intestine into enterocytes, where it is incorporated into chylomicrons and secreted into the circulation. Unabsorbed cholesterol and bile acids are excreted from the body in feces as neutral steroids and bile acids. The absorption of cholesterol from the intestine depends on the amount of cholesterol in diet and in bile, and on the absorption efficiency of cholesterol, which varies considerably interindividually, the usual range being 30 to 80 % (Grundy, 1983). Cholesterol absorption efficiency has been reported to regulate serum cholesterol levels in men, so that cholesterol absorption efficiency was correlated positively with serum and LDL cholesterol levels. Cholesterol synthesis takes place mainly in the liver, but nearly all human cells are capable of synthetizing cholesterol. The initial step in cholesterol synthesis is the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) into mevalonic acid by HMG-CoA reductase (Lennernas et a., 1997), which is the rate limiting enzyme in cholesterol synthesis (Biardi et al., 1997). Mevalonic acid is then transformed into squalene, and after many stages finally into cholesterol. Some of the cholesterol precursor sterols that are part of the chain leading from squalene to cholesterol are present in detectable amounts in serum, particularly desmosterol and lathosterol. They are transported in serum by lipoproteins similarly to cholesterol. The ratios of these precursor sterols to cholesterol are correlated positively with cholesterol synthesis and negatively with cholesterol absorption efficiency. Thus, the precursor sterol ratios can be used as indicators of cholesterol synthesis rate. Cholesterol synthesis in the body is strictly regulated (Brown and Goldstein, 1986).

In cells, cholesterol esters are hydrolyzed to free cholesterol, which can be used as a constituent of cell membranes, in the biosynthesis of steroid hormones, or re-esterified for storage. Cholesterol entering the cell suppresses the cell's own cholesterol synthesis thus keeping up a homeostasis in the cholesterol content of the cell. In hepatocytes, cholesterol can also be secreted back to the blood in lipoprotein particles or it can be converted into bile acids or secreted as free cholesterol with bile acids to the bile. Cholesterol and triglycerides, which are insoluble in water, are transported in blood plasma via lipoproteins. The major density classes of lipoprotein particles include chylomicrons (density < 0.94 g/mL), very-low-density lipoproteins (VLDL, < 1.006 g/mL), intermediate-density lipoproteins (IDL,1.006-1.019 g/mL), low-density lipoproteins (LDL, 1.019–1.063 g/mL), lipoprotein (a)(Lp(a) 1.050-1.120) and high-density lipoproteins (HDL, 1.063-1.21 g/mL). In human plasma, lipids are transported as lipoproteins which consist of cholesterol esters and triglycerides in the hydrophobic core, and of free cholesterol, phospholipids and apolipoproteins on the hydrophilic surface.

2.1.1. Chylomicrons

Chylomicrons are very large lipoproteins that consist mainly of triglycerides and of minor amounts of cholesterol and phospholipids. Their major protein is apolipoprotein (apo) B-48, and they also contain apolipoproteins A, C and E. Chylomicrons are formed postprandially from dietary fats in the intestinal epithelial cells, and they enter the circulation via lymph. In the blood, triglycerides are rapidly hydrolyzed into free fatty acids by lipoprotein lipase. Some cholesterol and phospholipids, and the A and C apolipoproteins are transferred to HDL. The resulting particle, called a chylomicron remnant, is taken up by hepatic receptors.

A newly synthesized chylomicron particle is composed of a large core of triglycerides (85-92%) and cholesteryl esters (0.8-1.4%), surrounded by a surface layer of phospholipids (6-12%), free cholesterol (0.8-1.6%), and apo’s (1-2%) such as apo B-48, A-I, and A-IV). Chylomicrons are heterogeneous in size, and their diameter ranges from 80 to 800 nm.

Newly assembled chylomicrons move to the Golgi lumen from the endoplasmic reticulum in the enterocytes, from which they are released into the intercellular space by reverse pinocytosis, and are transported to plasma via the thoracic lymph duct. Chylomicrons are carried through lymphatic channels to the plasma, where they circulate, receive other apoproteins including apoCII from HDL, and interact with lipoprotein lipase (LPL), situated in highest concentrations in the blood vessels overlying adipose (fat) tissue and skeletal and cardiac (heart) muscle. Digestion of chylomicron triglyceride by LPL requires the presence of apoCII as an essential cofactor. Following digestion of most of the triglyceride in the chylomicron particle by LPL, the remaining chylomicron “remnant” receives apoE from HDL and is removed from the bloodstream through an interaction of the apoE on its surface with the LDL receptor, LDL receptor-related protein (LRP), and perhaps another unique chylomicron remnant receptor. Chylomicrons are metabolized very rapidly (half-life about 15 min.) in plasma by LPL, and are normally completely gone from plasma by 6-8 hours after a meal. The clinical assessment of plasma lipid levels is usually done with the individual fasting for 10-12 hours, after which time no chylomicrons should be present in plasma. Disturbances in removal of postprandial chylomicrons from plasma exposes the vascular walls to these lipoproteins suggesting direct involvement of these lipoproteins in pathogenesis of atherosclerosis. Normolipidemic patients with CHD are reported to have significantly higher levels of chylomicron remnants than normal subjects. Chylomicron remnants penetrate the arterial wall efficiently and are seletively retained at the site of lesion. Increased production of remnant particles by acyl-cholesterol acyltransferase (ACAT) may also be an important mechanism of he postprandial lipemia of CHD. Chylomicron remnants were found to be toxic to cultured smooth muscle cells and macrophages. Thus damage to smooth muscles and macrophages in the arterial wall may be yet another mechanism by which chylomicrons can cause atherosclerosis (Hamilton et al.,1998).

2.1.2. Very low density lipoproteins

(VLDL): is synthtized by the liver and intestines. The VLDL transports hepatic synthesized triglycerides and cholesterol and are the major vehicle for plasma triglycerides transport in fasting state. VLDL is about 30-80 nm in diameter and contains triglycerides (45-65%), phospholipids (15-20%), cholesterol esters and unesterified cholesterol (16-30%), and protein (6-10%). Lipoproteins in VLDL are apo B-100, apo C, apo E and traces of apo A and B-48. The molecular weight is 5-10 kD. The plasma triglyceride level doubled and reached its peak about 3 hours after intake of the meal in the population of healthy individuals. VLDL carries endogenously synthesized lipids from the liver. In the blood, lipoprotein lipase hydrolyzes triglycerides, and the size of the particles diminishes. As VLDL passes through circulation, triglyceride is removed by lipoprotein lipase. Apo C and apo E are transfered to HDL (Packard et. al., 1984). At the same time cholesterol esters are transfered from HDL to VLDL. This transfer is accomplished by cholesteryl ester transfer protein. Some of these particles, called VLDL remnants, are directly removed from the blood by LDL receptors on hepatocytes (Murdoch et al., 1995). VLDL surface remnants, especially phospholipids, are transported to HDL via the function of phospholipid transfer protein (PLTP). The remaining particles are transformed into IDL, and, through loss of apolipoproteins E and C and further hydrolysis of triglycerides by hepatic lipase, further into LDL. The VLDL now contains equal amounts of cholesterol and triglycerides and is called intermediate density lipoprotein (IDL). IDL has a short life and is converted to LDL by liver. VLDL particles carry five times more cholesterol per particle than do LDL and are responsible for delivering substantial cholesterol to the arterial wall. Chylomicrons are too large to be able to penetrate arterial wall but VLDL and chylomicron remnants with their small size may have greater propensity to traverse the arterial intima and induce foam cell formation. VLDL and chylomicrons are rich in triglycerides. Lipoprotein lipase avidly binds these triglyceride rich lipoproteins (TRL) to heparan sulfate which is abundant on the surface of many cells including macrophages found in arterial wall.

This binding encourages cellular uptake of cholesteryl esters from TRL, thus providing another mechanism for atherogenity of TRL. Hypertriglyceridemia is positively associated with plasminogen activator inhibitor (PAI-1), a plasma protein which inhibits fibrinolysis and may be partly responsible for CHD.

2.1.3. Low Density Lipoproteins (LDL)

LDL is the end product of VLDL metabolism. It is produced from small VLDL particles, larger VLDL particles being rapidly removed from the circulation. The production rate depends both on the rate at which VLDL is produced and the rate at which VLDL remnants and IDL are removed from the circulation via LDL receptors, and also on the lipolytic activity in the transformation of VLDL and IDL into LDL (Hevonoja et al., 2000). In addition, some LDL can be directly secreted by hepatocytes, especially in hyperlipidemic states (but also in normolipidemia). LDL is a spherical particle and has a diameter of 22-28 nm. It contains 35-45% of cholesterol esters and 6-12% of triglycerides in the core, and 20-25% of phospholipids, 6-10% of free cholesterol, and 20-25% of protein on the surface. The core is composed of some 1. 600 molecules of CE (long chain fatty acid) and 170 molecules of TG. The CE is the main lipid of the lipoprotein core with the most fatty acyl chain in these esters being linoleate. This core is shielded by a layer of PL (700 molecules), FC (600 molecules), and 1 molecule of apoB-100. The sole protein of LDL is apo B, and one LDL particle always contains one molecule of apo B. LDL consists of one single copy of apo B-100, and of large amounts of cholesterol esters and smaller amounts of free cholesterol, triglycerides and phospholipids. LDL is the main carrier of cholesterol in blood, and it is responsible for transporting cholesterol to peripheral cells. Apo B interacts with specific LDL receptors located on cell surfaces in many tissues, including the liver, and thus LDL particles are mainly removed from the circulation. ApoB-100 is a large (513 kDa), single chain glycoprotein composed of 4536 amino acid residues with a coding gene residing on the short arm of chromosome 2.

There is only one apoB-100 molecule in each LDL. ApoB-100 also is not transfered between lipoprotein particles during the metabolic conversion of VLDL into LDL. Plasma LDL is not a homogeneous lipoprotein population, but consists of multiple subclasses with differing size and density. The density and molecular weight of LDL particles are negatively correlated so that as the density of the particles increases, their weight and size decrease (Crouse et al., 1985). LDL derived from the VLDL-IDL lipolytic cascade is initially large and buoyant, and as a result of exchange of lipids between lipoproteins it becomes smaller and denser (Fisher et al 1980). Small dense LDL particles consist of relatively more protein and less cholesterol than bigger and more buoyant LDL particles (Teng et al 1983). Serum triglyceride levels are positively related with LDL density so that hypertriglyceridemia is often accompanied by small dense LDL particles. High serum total cholesterol concentration has been strongly connected with atherosclerosis in numerous studies (Keys et al 1970).

2.1.4. LDL mutation and binding

It is presumed that the apoB-100 binding site resides in the carboxyterminal portion of the molecule. However, the region assumed to be involved in LDL binding is not yet clear (Shireman et al.,1977). Serum level of LDL is dependent both on the LDL production rate and LDL's clearance from the circulation. LDL cholesterol level has been reported to have both a positive correlation with the production of LDL apo B and a negative correlation with the clearance of LDL from the circulation in some studies, while others have reported the level to be associated only with LDL production. The activity of LDL receptors affects the rate of clearance of LDL from the circulation but also its production rate, because VLDL remnants are taken up from the circulation by the same receptors, and when their activity is low, more VLDL is transformed into LDL. High serum levels of LDL cholesterol, i.e. hypercholesterolemia or type IIa hyperlipidemia, can be a result of elevated LDL production, low LDL clearance, or both.

Familial hypercholesterolemia (FH) is a condition caused by a defect in the gene encoding the LDL receptor (Goldstein et al 1995). Heterozygotes have about half the normal receptor activity, and homozygotes have practically no functional LDL receptors. Defective clearance during the whole length of the lipolytic pathway (VLDL ? IDL ? LDL) leads to high levels of LDL cholesterol in the blood (Shepherd and Packard, 1989); heterozygotes usually have two- to threefold higher LDL cholesterol levels than normal population. A far more common form of hypercholesterolemia is polygenic in origin and in this case the serum levels of LDL cholesterol are mildly to moderately high. High levels of LDL cholesterol can be associated with high serum triglyceride levels; this condition is called combined hyperlipidemia, or type IIb hyperlipidemia. Familial combined hyperlipidemia (FCHL) is a genetically heterogenic entity, and affected individuals in a family may have either hypercholesterolemia (type IIa), hypertriglyceridemia (type IV), or both (type IIb) (Grundy et al., 1987). The basic defect in FCHL appears to be overproduction of apo B, and serum apo B levels are characteristically elevated. Being the main carrier of cholesterol in blood, LDL is also the principal lipoprotein causing atherosclerosis.

2.1.5. High Density Lipoprotein (HDL)

HDL contains 16-25 % cholesterol and cholesteryl ester and 45-55 % protein mainly apo A-I, apo A-II and apo D. HDL is mostly synthesized in liver and intestine (Eisenberg et al.,1984). HDL has a diameter of 4-15 nm. However intestinal HDL does not contain apo C and apo E, which are synthesized in liver and added to intestinal HDL (Brewer et al., 1991). Apo C and apo E are required for metabolism of chylomicrons and VLDL. Nascent HDL consists of a discoid bilayer of apolipoprotein and free cholesterol. Enzyme lecithin cholesterol acyltransferase (LCAT) binds to the disc and converts surface phospholipid and free cholesterol to cholesteryl ester and lysolecithin.

Lysolecithin is transfered to serum albumin (Robinson et al., 1987). Thus HDL is involved in removal of unesterified cholesterol from lipoproteins and tissues. HDL lipoproteins are encoded by genes clustered on chromosome 11. Several disorders are associated with mutations of these lipoproteins, in particular apo A-I, apo A-IV, and apo C-III lead to defect in synthesis or the function of HDL and development of atherosclerosis. For example, apo A-I serves as a factor in enzyme LCAT which catalyzes the conversion of cholesterol to cholestryl ester, the form transported in the HDL particle. Some variants of apo A-I are inefficient in this function. Authorities vary in attaching importance of HDL vs. LDL as a risk factor for CHD. However the ratio of LDL-C to HDL-C or total cholesterol toHDL-C is accepted as extremely important indicator of atherogenesis. HDL has an important role in reverse cholesterol transport: first specific HDL subclasses (preâ-HDL) function as primary cholesterol acceptors and are able to remove cholesterol from peripheral cells, and after cholesterol esterification cholesterol esters are delivered from HDL to apo B-containing lipoproteins, which can be removed from the circulation by hepatic receptors. Numerous epidemiologic studies have conclusively demonstrated that elevated levels of HDL protect against CHD, and abnormally low level increases the risk of CHD. Hypertriglyceridemia leads to enrichment of HDL which is remodeled to form smaller particles, probably enhancing catabolism. Low levels of HDL are commonly seen in diabetic patients and may contribute to higher risk of CHD. HDL levels increase with estrogen replacement therapy in postmenopausal women. HDL subfractions are increasingly recognized as an important factor associated with cardiovascular health. HDL2 had the strongest association with CHD (Miller et al., 1975,Tall et al., 1986).

2.1.6. Lipoprotein (a) [Lp(a)]

Lp(a) particles are heterogenous in density and size, and consist of approximately one third phospholipids, one third cholesterol, and one third protein. The protein of Lp(a) is comprised of apo B-100 linked by disulfide bridge to apo A. Apo A, a unique protein, is glycosylated and the molecular weight varies from 300,000 to 800,000 daltons. Heterogeneity is due to the polypeptide chain polymorphism and the extent of glycosilation. When exposed to a sulfhydryl reducing agent, apo A can be removed from Lp(a) by ultracentrifugation resulting in free apo A and Lp (a-) particles. Lp (a-) is somewhat larger than LDL and contains more triglyceride and is covered completely by apo B-100. Plasma levels of Lp(a) are genetically controlled (Scanu et al., 1992). The distribution of Lp(a) level in population is profoundly skewed. The apo A gene is located on the long arm of chromosome 6 q and is closely linked to the plasminogen gene. Lp(a) is synthesized in liver.

Lp(a) and atherogenesis**:** Endothelial cell injury leads to the deposition of lipids in the subendothelium. Lp(a) and LDL are taken up by macrophages, resulting in formation of foam cells. At the same time endothelial injury leads to the deposition of platelets and fibrin in the subendothelium (Mahler et al., 1995). These events stimulate smooth muscle proliferation and formation of atheromatous plaques. Although both Lp(a) and LDL are found in plaques, Lp(a) is preferentially deposited because Lp(a) bind more avidly to matrix constituents such as fibrin, glycosoaminoglycan, and fibronectin. Cholesterol loading of macrophages also enhances Lp(a) uptake and further induces foam cell formation. Lp(a) promotes smooth muscle proliferation by inhibiting transforming growth factor ß which is an inhibitor of smooth muscle growth (Seman et al., 1999).

2.2. ATHEROSCLEROSIS

Epidemiological studies have identified several risk factors for atherosclerosis. The classical risk factors explain only a part of the epidemiological features of atherosclerotic diseases.

Major risk factors for atherosclerosis:

1. Elevated cholesterol level (especially LDL cholesterol)
2. Low HDL cholesterol
3. Hypertension (high blood pressure)
4. Diabetes
5. Age: men > 45; women > 55
6. Family history of premature atherosclerosis

Other contributing factors:

elevated triglycerides (triacylglycerols)
elevated Lp(a),
obesity;
sedentary lifestyle;
high stress levels;
homocysteine,
fibrinogen,
C-reactive protein.

Despite the knowledge of many risk factors for atherosclerosis, its complications, such as coronary heart disease (CHD) remain the leading cause of death in many countries. Atherosclerosis is to be a degenerative disease (Steinberg et al., 1989). The relative abundance of the different plasma lipoproteins appears to be of primary importance, as raised levels of atherogenic lipoproteins are a prerequisite for most forms of the disease. The lesions of atherosclerosis occur principally in large and medium-sized elastic and muscular arteries.

The earliest recognizable lesions of atherosclerosis, the so-called fatty streaks, are common in infants and young children (Stary et al. 1989). Small fibrofatty plaques are common in young adults and extensive atheroma lesions from early middle age on. These lesions increase in number with age. The walls of arteries are composed of three well-defined layers called tunics. The layers, from the lumen outward, are tunica intima, tunica media and tunica adventitia. The intima is composed of a single layer of endothelial cells seated on a specialized extracellular matrix (ECM), the basement membrane. The endothelium, with its intercellular tight junctional complexes, functions as a selectively permeable barrier between blood and tissues. Endothelium is a monolayer inner lining of the vascular system covering almost 700 m^2 and weighing 1.5 kg.The media contains densely packed smooth muscle cells (SMC) arranged in single or multiple layers, surrounded by an ECM of collagen types I and III, fibronectin and chondroitin/dermatan sulphate proteoglycans. The adventitia consists of fibroblasts, SMC, the vasa vasorum and the nerves, all placed in a loose layer of thick bundles of collagen and elastic fibres. The atherosclerotic lesion is the most common acquired abnormality of blood vessels. The exact mechanism of atherogenesis is still unclear though there are three components, cholesterol, infection and inflammation enrolled in the genesis of atherosclerosis. There is a sequence of events leading to plaque formation.

2.2.1. Lipid infiltration theory

The initial step involves modification of plasma LDL that has entered the intima to create a form, which is a mild inflammatory mediator. This modified lipoprotein invokes monocyte adhesion to, and migration through, the intact endothelial surface (Berliner et al., 1995). Within the intima, lipoproteins are further modified by oxidation and are taken up avidly by the monocytes to become lipid-filled foam cells. Interaction of oxidized LDL (OxLDL) with macrophage-derived foam cells is one of the key events in the development and progression of atherosclerosis.

The appearance of lipid-laden macrophages in the vessel wall is one of the characteristic features in the development of atherosclerosis (Brown et al., 1983). During atherosclerosis, monocytes infiltrate and accumulate in the arterial wall and subsequently transform to foam cells after the uptake of modified lipoproteins, such as oxidatively modified LDL (OxLDL). OxLDL is suggested to play a key role in the transformation of monocytes and macrophages into resident foam cells in the atherosclerotic plaque. OxLDL may also act as a chemotactic agent in the atherosclerotic plaque, which results in an increased infiltration of monocytes into the vessel wall. The continuous interaction of modified lipoproteins with macrophages in the atherosclerotic plaque may therefore be of importance for the progression of the disease. It has subsequently been shown that trapped LDL does indeed undergo modification, including oxidation, lipolysis, proteolysis and aggregation, and that such modifications contribute to inflammation as well as to foam-cell formation. One of the modifications most significant for early lesionormation is lipid oxidation as a result of exposure to the oxidative waste of vascular cells. Such modifications initially give rise to ‘mini-mally oxidized’ LDL species that have pro-inflammatory activity but may not be sufficiently modified to be recognized by macrophage scavenger receptors. Mice lacking 12/15-lipoxygenase have considerably diminished atherosclerosis, suggesting that this enzyme may be an important source of reactive oxygen species in LDL oxidation. Lipoxygenases insert molecular oxygen into polyenoic fatty acids, producing molecules such as hydroperoxyeicosatetraenoic acid (HPETE), which are likely to be transferred across the cell membrane to ‘seed’ the extracellular LDL. The pathological processes of atherosclerosis are thought to be initiated by interaction between modified forms of LDL, monocyte-derived macrophages, T cells, and the normal cellular elements of the arterial wall. Scavenger receptors, which are expressed at the cell surface of macrophages, can mediate the uptake and internalization of modified lipoproteins into the cell. So far, 4 different types of receptors for OxLDL have been identified.

Two isoforms of a scavenger receptor class A with similar binding properties are characterized, type I and type II. Isoforms recognize chemically modified lipoproteins, eg. acetylated LDL, oxLDL, malondialdehyde-conjugates of either LDL or albumin, and polyanions. Both isoforms have a molecular mass of 220 kD and contain an ? -helical and a collagenous-like coiled coil domain. The type I scavenger receptor contains a C-terminal cysteine-rich domain, which is absent in the type II scavenger receptor. In addition, specific binding of OxLDL to CD36, a 94- to 105-kD protein membrane glycoprotein, is described. CD36 is a member of the class B scavenger receptors and is predominantly expressed on monocytes, platelets, and microvascular endothelial cells (Endeman et al., 1993, Febbraio et al., 2001). The recruitment and migration of monocytes and T cells to the arterial intima from blood stream to the vessel wall is believed to be the initial step in atherosclerosis and largely governed by adhesion molecules and chemokines. These inflammatory processes can ultimately lead to the development of complex lesions, or plaques, within the innermost layer of an artery wall. These plaques protrude into the arterial lumen, narrow the vessel lumen and reduce the flow of blood to the tissue/organ it supplies. Plaques that are likely to rupture and stimulate thrombogenesis are termed unstable. Evidence suggest that enzymes released from macrophages eat away at the thin, lipid-rich and foam cell-rich peripheral margins of the plaque, precipating plaque rupture, which in turn leads to thrombosis and results in the acute clinical complication of myocardial infarction (MI) or stroke. Numerous pathophysiologic observations in humans and animals led to the formulation of the response-to injury hypothesis of atherosclerosis. This hypothesis initially proposed that endothelial denudation or dysfunction was the first step in atherosclerosis. The hypothesis states that the protective, inflammatory response followed by the formation of a fibroproliferative response with time and continuing insult may became excessive. Possible causes of endothelial dysfunction include elevated and modified LDL; ICs; free radicals caused by cigarette smoking, hypertension, and diabetes mellitus; elevated plasma homocysteine concentrations; infectious microorganisms; and combinations of these factors.

Mast cell and neutrophils have also been proposed to play a role in the disease progression. In the coronary artery, the majority of mast cells are found in the outer layer of adventitia (ten times as many as in the intima) (Stary et al., 1990). Mast cells were recently shown to have accumulated in coronary plaques. Activated mast cells, found in the shoulder region and adventitia of advanced and complicated plaques, secrete enzymes such as neural protease and histamine, which may degrade extracellular matrix, cause rupture of atheromatous plaque and induce acute coronary syndrome.

2.2.2. The infections theory

Infections are known to increase the blood viscosity, cause hypercoagulability, and influence the serum lipid profile. Epidemiological studies have detected a correlation between the incidence of atherosclerotic complications and the presence of infectious microorganisms. Chlamydia pneumoniae is a common pathogen in respiratory infections and has been detected in atherosclerotic lesions (Hu et al., 1999). It can survive intracellularly in macrophages, which could be important for transportation of C. pneumoniae in the human organism. It has been proposed that C. pneumoniae aggravates atherosclerosis by activating macrophages to secrete tumor necrosis factor and metalloproteinases and/or by eliciting production of antibodies that cause endothelial cytotoxicity. Further studies will be necessary to clarify the association between infectious microorganisms and atherosclerosis in humans.

2.2.3. Inflammation theory

Atherosclerosis is characterized by the recruitment of monocytes and lymphocytes, but not neutrophils, to the artery wall. A triggering event for this process is the accumulation of minimally oxidized LDL, which stimulates the overlying ECs to produce a number of pro-inflammatory molecules, including adhesion molecules and growth factors such as macrophage colony-stimulating factor (M-CSF) (Ross et al., 1999).

The biological activity of minimally oxidized LDL is contained primarily in its phospholipid fraction, and three active oxidation products resulting from the scission or rearrangement of unsaturated fatty acids have been identified. Much interest has been focused on the putative role of autoantibodies. Immune complex formation is a highly efficient mechanism of host defense directed to eliminate the antigen and favor an adequate immune-response. Both autoantigens and exogenous antigens can trigger the formation of ICs. OxLDL is immunogenic in nature and has the ability to induce autoantibody formation (Esterbauer et al. 1992). Concentrations of plasma autoantibodies to oxLDL correlate with both the accumulation and depletion of arterial oxLDL. Antibodies binding to MDA-LDL (an epitope on oxLDL) are predictive of the rate of atherosclerotic disease progression. Several studies suggest that high levels of autoantibodies against oxLDL were predictive of atherosclerosis and its complications. The highest concentration of LDL receptors occurs in the liver. This receptor-mediated uptake is tightly regulated: once the cell has obtained the cholesterol it needs, it shuts down LDL receptor synthesis and so prevents intracellular accumulation of cholesterol (Brown and Goldstein 1986). Any excess of LDL particles not taken up by cells is rapidly removed by the lymphatic receptors. To study this interaction, we isolated macrophage-derived foam cells directly from in vivo developing atherosclerotic lesions and studied the expression of receptors for OxLDL during the atherosclerotic process.

2.2.4. Plaque formation

It is concluded that the expression of high levels of scavenger receptors for OxLDL may mediate the extensive lipid accumulation in macrophage-derived foam cells that is observed in advanced lesions. The histomorphometric characteristics associated with plaque disruption include the following: (a) a large, soft lipid core; (b) a thinned-out fibrous cap; (c) active infiltration by inflammatory cells into the plaque and fibrous cap; and (d) increased neovascularity in the plaque. Mature atherosclerotic plaques are comprised of two main components: a lipid-rich core and an extracellular matrix consisting of collagen and other matrix proteins.

Some plaquematrix is often organized as a lipid-poor, collagen-rich protective fibrous cap that separates the core from the lumen. The greater the amount of extracellular matrix, the more “stable” the plaque. More than 70% of a typical stenotic coronary plaque consists of extracellular matrix. In contrast, the lipid core consists of soft, usually hypocellular and a vascular, atheromatous plaque which is composed primarily of extracellular lipids (e.g., cholesterol and its esters) and increases its vulnerability to disruption. Vulnerable plaques are characterized by a thin fibrous cap overlying a macrophage-rich atheromatous core. Regional macrophages overexpress matrix degrading enzymes such as matrix metalloproteinases (MMPs) and pro-thrombotic molecules including tissue factor. Both can contribute to plaque vulnerability and thrombogenecity resulting in the onset of acute coronary events (Yano et al.,2000).

2.3. Oxidative stress, antioxidants and vitamins

Oxidative stress plays an important role in the patho-physiology of atherosclerosis. Lipid peroxidation, which is often developing during oxidative stress, either on the level of cell membranes (not only outer cell membrane, but also within mitochondria and endoplasmic reticulum) or on the level of macromolecules (lipoproteins, in particular low density lipoproteins, LDL) is considered as harmful event that might hardly be reverted and will lead, as a chain reaction, to the spread of oxidative stress (Panasenko et al., 1991, Jialal and Fuller.,1995). Producing various ROS in lipid peroxidation (either of saturated or of polyunsaturated fatty acids) will lead to production of the “end products” of lipid peroxidation reactive aldehydes. The most relevant in current biomedicine are malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (Irribaren et al.,1997). These aldehydes are able to “simulate” complex oxidative stress and its harmful consequences, hence it is commonly accepted that these molecules are considered as “second toxic messengers” of oxygen free radicals. Metal ions like Cu^{+2} or Fe^{+2} are frequently used to initiate LDL oxidation in vitro, since the resulting oxyLDL has similar biological activation to that oxidized in vivo (Esterbauer et al., 1992).

The kinetics of Cu^{+2} induced oxidation is well characterized and different approaches to study oxidation have been used. Lipid peroxidation (LPO), especially LDL oxidation is probably the most intensely investigated phenomenon of OS. LDL oxidizes in three consecutive phases. During the lag phase antioxidants are consumed and only minimal production of oxidation products can be observed. After the entire consumption of chain breaking antioxidants oxidation products like free radicals, peroxides and aldehydes are produced during the propagation phase. The generation of oxidation products shows the characteristics of a chain reaction. During the last phase, the degradation or decomposition phase, this chain reaction breaks down due to the lack of oxidizable substances. LDL and its carrier protein Apolipoprotein B 100 (ApoB 100) are degraded to smaller particles. Methods for measurement of OS and/or LPO events include direct methods, which detect free radicals, conjugated dienes or other LPO products, and indirect methods, which measure antioxidants or LPO events by degradation of e.g. oxidation sensitive dyes. Measurement of conjugated dienes: This method is based on the fact that during lipid peroxidation polyunsaturated fatty acids (PUFAs) develop double bonds which are separated by a single bond (= - =), commonly addressed as conjugated dienes. This diene formation can be measured online at 234 nm. Its main advantage is that LPO can be followed online. Measurement of peroxides: Several sufficient methods for peroxide detection have been described. Among the most common there are luminometric and colorimetric detection of hydroperoxides and the detection of lipid hydroperoxides by a complexometric method. The main advantages of the methods are that they are relatively easy to perform. Their main disadvantages are that they can measure only parts of the spectrum of peroxides in a pH dependent manner.

Measurement of thiobarbituric acid reactive substances (TBARS): TBARS are mainly aldehydes and the most important aldehyde in this measurement is malonic dialdehyde (MDA). The measurement of TBARS is a relatively simple colorimetric method.

Antioxidants have been experimentally shown to protect against the alterations. Diets rich in antioxidant substances, such as beta-carotene, alpha tocopherol (vitamin E) and ascorbic acid (vitamin C), are consumed by populations with a low incidence of coronary atherosclerosis, which supports the oxidation theory. Flavonoids, substances have an anti-inflammatory and antioxidant effect (Hollman et al., 1996, Hollman and Katan, 1997). In various studies they have been shown to reduce the incidence of atherosclerotic lesions. Probucol, a lipid-lowering agent with a powerful antioxidant effect has been proven to reduce atherosclerosis in experimental animals and the coronary restenosis. The increased VLDL, triglycerides, atherogenic small dense LDL cholesterol and the diminished amount of the anti-atherogenic, antioxidant anti-inflammatory, high density lipoprotein cholesterol would reduce the natural antioxidant reserve. This combination supports an increase in redox stress in addition to the previously discussed FFA toxicity. This also tends to support the oxidation, glycation and glycoxidation of existing lipoproteins which results in increased ROS and redox stress. Lipoproteins have the function of transporting lipids throughout the body. Low density lipoproteins are responsible primarily for the transport of cholesterol with the protein moiety involved: apolipoprotein (Apo) B 100. Very low density lipoproteins are responsible for the transport of triglycerides with the protein moiety involved: Apo E. High density lipoproteins are responsible for reverse cholesterol transport and play an important role in being a naturally occurring potent anti-inflammatory and antioxidant agent with the protein moiety involved: Apo A. It is the protein moiety of the lipoproteins that is modified by the processes of oxidation, glycation, and glycoxidation with a resultant increase in redox stress and the production of ROS. Furthermore, the modification of the protein moiety is responsible for their retention within the intima, inducing atherogenesis. In addition, these toxic metabolic products are thought to cause the complications of insulin resistance, obesity, cardiovascular disease and T2DM by creating cellular dysfunction and, in time, promoting programmed cellular death (lipoapoptosis).

In the normal state, FFA delivery to non-adipose tissue is closely regulated to its need for fuel. FFAs normally rise during exercise and fasting in order to meet metabolic requirements and thus, homeostasis is maintained. However, as a result of over-nutrition (western diet), the FFA influx may exceed FFA usage and FFA non-beta oxidation ensues. These non-mitochondrial FFA metabolites which are responsible for injuring cells, resulting in lipoapoptosis, include triglycerides, ceramide, and products of lipid peroxidation. Autoxidation occurs at the site of the protein component embedded within the LDL cholesterol particle resulting in glycated LDL and glycoxidated LDL cholesterol which contribute to its retention just as oxidized LDL is retained within the intima which initiates and sustains atherogenesis. Native LDL is not atherogenic and is not retained within the intima; however, if it becomes modified by oxidation, glycation, glycoxidation or homocysteinated, it becomes modified and retained (trapped to adjacent glycosaminoglycans and structural glycoproteins) to initiate and maintain an atherogenic process within the intima. Hypertriglyceridemia certainly plays a role in toxicity regarding the development of redox stress, not only its role in lipotoxicity and FFA toxicity discussed previously, but independently as its own marker of toxicity. There is a close association of hypertriglyceridemia and the atherogenic small dense LDL cholesterol particles which are more likely to be oxidized and contribute to redox stress.

2.4. Animal models

The general physiology of rabbits is similar to humans, and therefore, the rabbit has been used as a model for human diseases with some frequency. The rabbit rapidly develops severe hypercholesterolemia leading to premature atherosclerosis in response to dietary manipulation. The rabbit is an important model for the study of the relationship between plasma cholesterol metabolism and atherosclerosis (Hodis et al.,1991, Juhel et al.,1997).

The major consequence of hypercholesterolemia in the rabbit is the rapid development of atherosclerosis (Lee et al., 1994). One of the earliest events in lesion development is a focal increase in arterial concentrations of low density lipoproteins (LDL). After less than two weeks on the cholesterol diet, subendothelial deposits of extracellular lipid and cytosolic lipid droplets within vascular smooth muscle cells appear (Restori et al. 1990). After just one month, fatty streaks are detected in the aorta which progress quickly to raised lesions containing macrophage-derived foam cells. During the next three to six months, these sites progress to more complex fibrous plaques that accumulate both intracellular and extracellular lipid deposits. The plaques become advanced atheromatous lesions characterized by an extensive distribution of smooth muscle cell-derived foam cells, collagen fibers, necrotic debris, and cholesterol crystals. Investigators demonstrated that laboratory chow supplemented with less than 2% cholesterol reproduced the effect, rapidly leading to plasma cholesterol concentrations that can exceed 2.000 mg/dl. This response could be enhanced by including up to 10% extra fat in the diet, with saturated fats increasing both circulating cholesterol levels and the extent of lesion development. In the rabbit, lesions are distributed predominantly in the aortic arch and thoracic aorta, at the origins of intercostal arteries, and to a lesser extent in the abdominal aorta. The involvement of the intercostal ostia may be due to a higher vascular permeability of LDL and to turbulent blood flow patterns at these locations. All lesion sites in the aorta are characterized by an increased binding of LDL to collagen and glycosaminoglycans in the vascular wall. The retention of lipoproteins by the extracellular matrix may facilitate their oxidative modification, thereby triggering local cellular responses that include the release of cytokines, the recruitment of lymphocytes and monocytes, expression of cell adhesion receptors, the retention of macrophages, and the migration and proliferation of smooth muscle cells. Supplementing the diet with cholesterol rapidly results in a marked increase in the production of cholesteryl ester-rich, beta-migrating very low density lipoproteins (beta-VLDL) by the liver and intestine. The VLDL become the major class of plasma lipoproteins.

Subsequent clearance of the beta-VLDL by the liver is reduced due to a downregulation of cell-surface lipoprotein receptors and the saturation of the remaining receptors. Two additional factors exacerbate the response of the rabbit: the relatively efficient absorption of dietary cholesterol and limited hepatic conversion of cholesterol to bile acids. The beta-VLDL, including chylomicron remnants, that accumulate in the circulation are highly atherogenic. Plasma levels correlate closely with the extent of lesion development. Important genetic variants of the New Zealand White rabbit have been identified that confirm the link between plasma cholesterol and atherosclerosis. An important model for human familial hypercholesterolemia, the Watanabe heritable hyperlipidemic (WHHL) rabbit, has defective LDL receptors. Thus, LDL accumulate in plasma to high levels,with homozygotes having plasma cholesterol levels in excess of 400 mg/dl. The WHHL rabbit develops complex fibrous lesions that are rich in foam cells. In these rabbits, the strongest predictor of aortic atherosclerosis is an elevated level of IDL (Rosenfeld et al.,1990).

Rabbits are heavily used to produce copious quantities of polyclonal antibodies. The procedure involves injecting the animal with antigen with adjuvant several times. There are some adjuvants used. These adjuvants include; Freund's Complete and incomplete, RIBI, TiterMax, and others. The animals immune system then develops antibody, which is taken by blood withdraws. The serum is harvested and the antibody extracted from the serum. It is then used as a reagent.

2.5. Hypocholesterolemia in cancer and in neuronal degeneration

Targeted drug delivery to cancer cells has a great potential to enhance the chemotherapeutical efficacy for treatment of many types of human malignant cancers. One of the targeting approaches is based on the high demand of cholesterol by the rapid dividing cancer cells (Markel and Brook, 1994, Nakagawa et al., 1995). Cancer cells usually grow very aggressively and need significant amount of exogenous cholesterol (in its ester form) for their proliferation and cell membrane construction. Many cancer cells show elevation in LDL (low density lipoprotein) receptor expression and, thus, higher consumption of LDL. LDL contains about 1500 molecules of cholesterol esters per LDL particle and functions as the main carrier of cholesteryl esters in blood circulation. Many different epidemiological studies discuss the relation between the high risk for cancer mortality and low blood cholesterol in patients with cancer. An enhanced receptor-mediated uptake of LDL by tumor tissue in vivo was demonstrated in an animal model and, subsequently, by solid tumors in vivo in humans. Accordingly, LDL particles have actually already been used “as a carrier for toxic compounds in order to kill tumor cells with high LDL receptor activity another multi-ligand receptor type, the "macrophage scavenger receptors" (Ponty et al., this group of receptors could well account for the observed preferential uptake of LCM by neuro-injury sites as well. (Since macrophage infiltration/proliferation is significantly increased in response to neuro-injury in the CNS). Similarly, these "macrophage scavenger receptors" participate in the pathogenesis of neuronal degeneration observed in aging and Alzheimer's disease, and accordingly such scavenger receptors have been identified in the literature as potential therapeutic targets in Alzheimer's disease as well as in atherosclerotic lesions.

2.6. Measurement of lipoproteins and cholesterol

The association of lipoprotein classes defined by Svedberg floatation intervals as assessed in the analytic ultracentrifuge. Intervals Sf 0-12 (LDL), Sf 12-20 (IDL), Sf (20-100) (VLDL) and Sf 100-400 (VLDL) were all noted to be associated with atherosclerosis, HDL2 (F 3.5-9.0), HDL3 (F 0-3.5) have antiatherogenic effects (Ewing et al., 1965, Lindgren et al.,1969, Chapman et al., 1988) . Preparative density gradient fractionation methods have been described for fixed-angle, vertical, swinging-bucket, and zonal rotors. The fixed angle rotor method has the advantage of giving a complete lipoprotein profile in a single spin. Separations may be made more commonly, in discontinuous (stepwise) gradients. Bands can then be recovered based on rate of flotation (non-equilibrium) or after fractions reach their equilibrium densities. Fractions may be recovered by pipetting from the top of the tube or by puncturing the tube and collecting the effluent from the bottom in appropriate fractions. The isolated fractions are available for analysis and are usually quantified in terms of the cholesterol content and sometimes the amounts of other lipids and apolipoproteins or total protein. LDL is not a homogeneous category of lipoproteins but consists of a set of discrete subspecies with distinct molecular properties, including size and density. In normal subjects, at least three major LDL subspecies can be identified: LDL-I is the largest and least dense, and, the smallest, LDL-III, is the most dense. Analysis of LDL subspecies is made possible by numerous techniques, including gradient gel electrophoresis, which separates LDL particles on the basis of their differing size, and ultra-centrifugation, which separates the particles on the basis of their differing density. In most healthy people, the major subspecies are large or buoyant, whereas the smaller denser LDL subspecies are generally present in small amounts.

Based on the relative density obtained in the analytic ultracentrifuge (ANUC), the more dense, relatively cholesterol-poor form is termed HDL3 (1.125 to 1.21 G per mL) and the less dense, relatively cholesterol-rich form is termed HDL2 (1.062 to 1.125 G per mL). The small LDL pattern B trait is linked to several metabolic issues that help explain it's atherogenicity and has been termed the atherogenic Lipoprotein Profile (ALP). Pattern B is a term used to describe individuals with a predominance of small LDL particles compared to pattern A, which describes individuals with predominately large LDL particles. Small LDLs are able to infiltrate the arterial wall approximately 40 to 50% faster than large LDL particles, these particles are more susceptible to oxidative damage, and the HDL subclass that plays the most powerful role as an antioxidant, HDL2b, is reduced in LDL pattern B subjects. Evidence that LDL heterogeneity is clinically important in determining arteriographic change over time comes from several investigations. The Monitored Atherosclerosis Regression Study reported that significant arteriographic benefit was evident in CAD patients with mid-density LDLs but despite an equal LDLC reduction, patients with predominately dense, or buoyant LDLs revealed no significant arteriographic benefit. The Stanford Coronary Risk Intervention Project has revealed that individuals with predominantly dense LDLs in the control group, had an approximate 2-fold greater rate of arteriographic progression, but with multifactorial risk intervention, they did significatly better than patients with predominantly buoyant LDL in regard to arteriographic benefit. Other lipoproteins, such as Lp[a] and IDL, are also involved in the development of CHD. Clinical interest in Lp[a] has been stimulated by numerous reports linking increased concentrations of plasma Lp[a] with an increased incidence of CHD. The importance of IDL cholesterol in the development of CHD has been measurement of TC alone may not be adequate to identify subjects at risk for CHD. An individual with normal or near-normal levels of TC may still be at risk because of low HDL cholesterol, elevated Lp[a] IDL cholesterol.

Therefore, measurement of the distribution of cholesterol among all lipoproteins lipoprotein cholesterol profile, in addition to the TC and LDL cholesterol, is desirable in order to assess risk for CHD accurately. The importance of IDL cholesterol in the development of CHD has been demonstrated by the Type II Coronary Intervention Study of the NHLBI (National Heart, Lung and Blood Institute). Increased CHD risk may be also related to increased concentrations of IDL in familial dysbetalipoproteinemia and familial combined hyperlipidemia patients. Thus, measurement of cholesterol concentrations in lipoproteins such as Lp[a] and IDL, in addition to LDL and HDL, may assist in the identification of a greater number of subjects at risk of CHD. Determination of total, HDL, LDL, IDL, and VLDL cholesterol and total triglycerides by enzymatic and spetrophotometric methods are often used in lipid laboratories (Allain et al., 1974). The most common enzymatic mehod employs at the cholesterol measurement the Trinder reaction, which includes the breakdown of cholesterol esters to free cholesterol by cholesterol esterase. Cholesterol oxidase, in the presence of oxigen, oxidizes free cholesterol to form choles-4-ene-3-one and hydrogen peroxide. The hydrogen peroxide reacts with phenol and 4-aminoantipyrine in the presence of peroxidase to form a quinoneimine dye. The resulting colour is measured by photometry. The amount of dye formed, determined by its absorption at 500 nm, is directly proportional to the total cholesterol concentration.

The triglycerides measurement procedure involves the hydrolysis of triglycerides by lipase. The glycerol concentration is determined in a coupled assay that terminates in the formation of quinoneimine dye. The amount of dye formed, determined by its absoprtion at 500 nm, is directly proportional to the triglycerides concentration.

The need for cholesterol measurement in major lipoproteins such as LDL, and HDL, in addition to the measurement of TC, has recently received much attention because of the strong association of these lipoproteins with the development of CHD.

In clinical laboratories, the cholesterol profile is determined by measuring TG, TC, and HDL cholesterol using three different aliquots of the same plasma, and VLDL cholesterol is calculated using the formula: VLDL cholesterol = TG/5. The LDL cholesterol is estimated from the above three measurements using the Friedewald formula. This is not only an indirect value but also includes cholesterol values of Lp[a] and IDL. Determination of a cholesterol profile in a clinical determination of a cholesterol profile in a clinical laboratory usually involves independent cholesterol measurement for each lipoprotein class using different aliquots of the same plasma sample, often using the formula of Friedewald, Levy, and Fredrickson. Measurement of HDL subclasses has been an useful research tool in studies of lipoprotein metabolism, pathophysiology, and association with CHD risk. Inter-individual differences in HDL values are largely due to the larger and lighter HDL2; the smaller, more dense HDL3 fraction is relatively more consistent within and among individuals.

2.7. Radiotracers and in vivo imaging in Nuclear Medicine

Techniques such as magnetic resonance imaging (MRI), X-ray computed tomography (CT) nuclear imaging, optical imaging and ultrasound, positron emission tomography (PET) and single photon emission tomography (SPECT) are used to detect and monitor deseases. Imaging can be used to track specific molecular markers and selectively highlight internal organs, tumours or molecular processes. Many of these non-invasive technologies were originally developed for human use, but have recently been scaled down to allow high-resolution imaging of small animals. This is important, because as the genomic era provides us with better animal models of atherosclerosis or cancer and more specific therapeutics, better methods are needed to monitor atheroscerosis or tumour development in animals.

Single photon emission tomography (SPECT) — the detection and quantification of gamma-emitting radionuclides — is commonly used to track individual molecules or cells. In this approach, the molecule or cell of interest is labelled with a gamma-ray-emitting nuclide such as 99m Tc, 111 In, 123 I or 125 I, injected into an animal and followed isotope decaying with gamma-ray emission. In nuclear medicine, a radiolabelled compound the radiopharmaceutical is injected (in most cases intravenously) and the distribution of the radioactivity throughout the body is visualized using a dedicated gamma camera. For diagnostic imaging agents, technetium-99m is a frequently used radionuclide, because it has optimal nuclide properties (half-life of 6h and appropriate Gamma-energy of 140 keV). The ^{99}Mo/^{99m}Tc generator is based on transient equilibrium between the parent radionuclide ^{99}Mo (66h half-life) and the daughter radionuclide ^{99m}Tc (6h half-life). The generator makes this radionuclide routinely available. The ^{99m}Tc-radiopharmaceutical can easily be prepared in the clinic by means of prefabricated kits. So it is no surprise that more than 90% of routine in vivo imaging is performed with technetium-99m (Isaacsohn et al., 1986, Ginsberg et al.,1990, Iuliano et al.,1996). Identifying the radiotracers that localize in large superficial vessels, such as the carotid arteries and the feasibility of detecting lipid laden plaques using radiolabeled low density lipoprotein and external planar gamma camera imaging becomes a major challenge. Alternative approaches to image atherosclerotic plaques, may have been made by using radiolabelled polyclonal human immunoglobulin G, monocytes or platelets but non significantly improved the rate of detection in comparison to LDL. This is due to both the low level of plaque uptake of these radiopharmaceuticals and to their plasma clearance that is slover than the physical half-life of the isotope. These two factors lead to a low target/ background ratio and poor diagnostic accuracy. An good tracer should have a high and specific binding to atherosclerotic lesions and a fast plasma clearance.

In vivo detection of atherosclerotic lesion may be a good means to image atherosclerotic plaques using a receptor specific radiopharmaceutical to prepare and isolated modified lipoprotein fractions. Dynamic gamma camera imaging revealed a faster plasma clearance of oxidized–LDL than native-LDL. The rapid blood clearance of oxidized-LDL resulted as a consequence of more rapid uptake by macrophage rich tissue. Similarly, a faster clearance and higher uptake has been demonstrated for acetoacetylated, methylated, and oxidized-LDL in rabbits. More ox-LDL than n-LDL accumulated in the affected carotids or aorta artheries. Modified Tc- labelled LDL behaved like a trapped ligand in vivo and could be used, therefore, as a tracer to study and compare to native LDL distribution and to show accumulation of LDL or other modified lipoprotein fractions in abnormal tissue sites by nuclear scintigraphy (Leitha et al., 1993).

The mouse peritoneal macrophages take up low-density lipoproteins (LDL) which have been chemically modified by the acetylation of lysine residues (Ac-LDL). This uptake is mediated through a specific receptor known as the scavenger receptor. Ac-LDL therefore appear to have excellent potential for antiinfectious drug targeting. The radiopharmaceutical used for imaging infection and inflammation accumulates in the infectious/ inflammatory lesion due to the locally changed physiological condition. An infectious/ inflammatory focus is characterized by enchanced blood flow, enchanced vascular permeability, and influx of white blood cells. Radiopharmaceutical for infection imaging accumulate in infectious/ inflammatory foci due to these local physiological features of inflammation. In addition, scintigraphic imaging is an exelent nonivasive method of the infectious or inflammatory disease throughout the body. So radiolabelled LDL or other modified lipoprotein fractions offer possibilities to detect inflammation cells throughout tho body by scintigraphy (Manderson et al., 1989).

Overexpression of low density lipoprotein (LDL) receptors occurs in several cancer cell lines and offers also unique possibilities for tumor targeting by using radioactive labelled LDL (Leppula et al.,1995, Manker et al., 1994).

Tc-LDL imaging studies, injected into the cancer animals had the ability to target not only the primary tumor but also its metastatic sites.This notable enhances the potencial usefulness of radiolabelled LDL as a diagnostic tool and eventually as a therapeutic tool using for radiolabeling of beta radionuclide such as Re-188. Nuclear medicine images were obtained from cancer patients with lipid emulsion (LDE) similar to the lipid structure of native LDL. The emulsion (LDE) does not contain apolipoproteins but when injected into the blood circulation acquires them in contact with plasma lipoproteins (Ginsburg et al., 1982, Rensen et al., 1997). Since LDE can be manufactored from commercially available materials, it may be the adequate device to explore the LDL receptor-mediated endocytic pathwais as a gate to deliver drugs to cancer cells.

2.8. Lipoprotein macromolecules for radiopharmaceutical applications

The aim of any drug delivery system is to modulate the pharmacokinetics and/or tissue distribution of the drug in a beneficial way. Among the variety of delivery systems that have been devised over the years are many particulate-carrier systems; for example lipoproteins, microspheres, nanoparticles, micellular systems and liposomes. There are a number of basic reasons for using specic systems to deliver therapeutic agents (Versluis et al., 1999). These include the following. (1) Targeting of the therapeutic agent to a particular site of action in the body. (2) Maintenance of drug levels in the therapeutic range over an extended period of time resulting in increased duration of action and reduced frequency of administration. (3) Protection of the therapeutic agent from degradation until it reaches its therapeutic target.(4) Internalization of the therapeutic agent through interaction of the delivery system with the membranes of cells in the target tissue. (5) Facilitation of the administration of therapeutic agents with a short in vivo half-life. (6) Use of the delivery system to amplify the effects of the delivered agent. The targeting of treatment, reduction in dose and number of administrations required to achieve.

Lipoproteins are involved in other biological processes as well, including coagulation and tissue repair, and serve as carriers of a number of hydrophobic compounds within the systemic circulation. proteins called apolipoproteins compounds within the systemic circulation. It has been well documented that disease states (eg, AIDS, diabetes, cancer) significantly influence circulating lipoprotein content and composition (Vitols et al., 1990). Therefore, it appears possible that changes in the lipoprotein profile would affect not only the ability of a compound to associate with lipoproteins but also the distribution of the compound within the lipoprotein subclasses. Such an effect could alter the pharmacokinetics and pharmacological action of the drug. Lipoproteins and the implications of altered plasma lipoprotein concentrations on the pharmacological behavior of some compound. A number of different phospholipids are incorporated into the coat of the lipoprotein, the more common of which are phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and sphingomyelin. The most abundant of these phospholipids is phosphatidylcholine, which is also utilized as a substrate in the esterification of cholesterol to cholesteryl ester by the enzyme lecithin:cholesterol acyltransferase. The liposome system has a major advantage over competing colloidal carrier systems: it allows almost infinite possibilities to alter structural and physicochemical characteristics. This feature of flexibility enables the formulation scientist to modify liposome behavior in vivo and to tailor liposome formulations to specific therapeutic needs. Conventional liposomes are either neutral or negatively charged, terically stabilized (‘stealth’) liposomes carry polymer coatings to obtain prolonged circulation times. Immunoliposomes (‘antibodytargeted’) may be either conventional or sterically stabilized. For cationic liposomes, several ways to impose a positive charge are shown (mono-, di- or multivalent interactions). Water-insoluble polar lipids in an excess of water give rise to highly ordered assemblages, which eventually arrange themselves into a system of concentric, closed, bilayered membranes called liposomes.

Liposomes are formed spontaneously when amphipathic lipids are dispersed in excess water. The lipid molecules arrange themselves by exposing their polar head groups towards the water phase, while the apolar hydrocarbon moieties stick together in the bilayer thus forming closed concentric bimolecular lipid leaflets separated by aqueous compartments (Roerdink et al., 1987). Their design allows water soluble drugs to be encapsulated in the aqueous core of the liposome and hydrophobic/lipophilic drugs to be incorporated within the phospholipid bilayer. The high density lipoproteins (HDL) were found to be responsible for penetration into the liposomal bilayers, a process which was accompanied by the net loss of phosphatidylcholine from the liposomes to the HDL. Low density lipoproteins may also increase liposomal permeability, taking lipid components from the liposomal membrane, especially when large doses of liposomes are used. However, the incorporation of cholesterol, causing increased packing of phospholipids in the lipid bilayer, reduces the transfer of phospholipids to the HDL. The lipid molecules arrange themselves by exposing their polar head groups towards the water phase, while the apolar hydrocarbon moieties stick together in the bilayer thus forming closed concentric bimolecular lipid leaflets separated by aqueous compartments (Oku et al., 1994). Their design allows water soluble drugs to be encapsulated in the aqueous core of the liposome and hydrophobic/lipophilic drugs to be incorporated within the phospholipid bilayer. Pharmacokinetics, tissue distribution and pharmacological activity for a number of drugs which are incorporated into liposomes are influenced by their interaction with serum lipoproteins. For most hydrophilic compounds intravenous administration results in an instantaneous distribution of the compound within the bloodstream. This is a consequence of homogenous mixing of the compound with the aqueous components of the bloodstream.

However, when compounds are hydrophobic in chemical nature or incorporated into lipophilic carriers (liposomes), their distribution within the bloodstream is not instantaneous.

The interaction of these compounds with non-aqueous components of the bloodstream, including lipoproteins, appears to be an explanation for this observation (Wasan et al., 1998).
The understanding the mechanism(s) of the lipoprotein-liposome interactions are of fundamental importance to learning more about the pharmacokinetic behaviour and pharmacological effect of liposomal products.

3. AIMS OF THE STUDY

Atherosclerosis and tumor targeting by radiopharmacons are very important aims in nuclear medicine. LDL acts as trapped ligand in vivo and should be good tracer for the scintigraphic studies of atherosclerosis and cancer. We have the novel use of both preparative and analytical ultracentrifugation along with gamma scintigraphy (Technetium labelling) to investigate the distribution of low density lipoproteins in animal models. In vivo detection of atherosclerotic lesion may be a good means to image atherosclerotic plaques using receptor specific lipoprotein fractions. One of targeting approaches is based on the high demand of cholesterol by the rapid deviding cancer cells with LDL targeted cancer cells via the elevated LDL receptors. To match of 99m Tc labelled lipoprotein fractions with a half-life of 6 h is attractive, since it is readily and economically available through a 99mMo/99mTc generator system.

Thus, the aims of this study were:

1. Construction of a discontinuous density gradient ultracentrifugation in an fixed angle rotor which permits the separation of the major lipoprotein classes (VLDL, IDL, LDL, Lp(a), HDL) of a whole serum sample and the determination of chemical compositions of lipoproteins by spectrophotometry and to developed new analytical ultracentrifuge micro-methods for the investigation of serum lipoproteins from ultracentrifuge Schlieren scans using special designed cells and special written software. With these method to gain insight of profiles in hyperlipoproteinemia of human and animal samples allowing comparison of serum lipoprotein concentrations, to study antihyperlipidemic effects of flavonoids in a herbal compound, to investigate the interaction of lipoprotein macromolecules with other molecules, and to obtain lipoprotein fractions to radiolabelled procedures.

2. Working out lipoprotein modification and radiolabelling procedures: oxidize lipoprotein fractions by chemical agents, and by gamma irradiation, develop efficient radiolabelled methods of lipoproteins by technetium, study the radiochemical purity and radiochemical stability of lipoproteins by technetium.

3. Adjust rabbit atherosclerotic model by cholesterol feeding, adjust nude mice tumor bearing model by human tumor cells setting, and the verification of animal models by pathological examinations

4. Develope a rapid and reproducable method to obtain n-LDL and ox-LDL suitable for radiolabelling with the versatile isotope 99mTc, study the biodistribution and uptake of both native and oxidized LDL by atherosclerotic plaques in rabbit hypercholesterolemic model, study the biodistribution and uptake of lipoprotein fraction by tumor cell in mice model and in spontaneous developed cancer in dogs.

5. Comparison of modified lipoptotein fractions for the use of radiopharmacy, study the uptake of lipoprotein fraction by mononuclear cells in vitro.

4. METHODS

4.1. Measurement of lipids

Total cholesterol (TC) is an obligatory blood (serum, plasma) measurement in the surveys. Wherever possible, samples should also be analysed for HDL-C and LDL-C. Other optional but complementary measurements might include, e.g.: triacyglycerols (triglycerides) and lipoprotein typing. For serum preparation, blood samples are allowed to clot at not abowe 20°C usually for up to one hour before centrifugation. Blood specimens should be centrifuged at a temperature of not more than 20°C. Centrifugation can be made at a minimum of 1500 G for at least 10 minutes to separate serum from the clot. After centrifugation, the serum/plasma should be promptly separated from clot or cells and transferred to a clean tube. Samples can be stored for up to four days at +4°C. If analyses of TC cannot be performed within 4 days the serum or plasma samples should be immediately stored at -20°C or lower in tightly stoppered glass vials.

Total cholesterol values reflect the total amount of cholesterol in the lipoproteins, and are determined by chemical or enzymatic methods. The specimen used may be whole blood, serum or plasma using either heparin or EDTA. Currently the majority of cholesterol test systems available utilize enzymatic methodology based on the use of coupled enzymatic reagent systems and various chromogens. The most common enzymatic method employs the Trinder reaction which includes the breakdown of cholesterol esters to free cholesterol by cholesterol esterase. Cholesterol oxidase, in the presence of oxygen, oxidizes free cholesterol to form cholest-4-ene-3-one and hydrogen peroxide. The hydrogen peroxide reacts with phenol and 4-aminoantipyrine in the presence of peroxidase (POD) to form a quinoneimine dye. In the most popular methods, phenol or other chromophores may be substituted.

Calibration materials should have concentrations around the decision levels. For total cholesterol appropriate calibrator levels range from 200 to 300 mg/dL. Two levels of controls are recommends, one in the normal range (175-200 mg/dL), and one near the concentrations for intervention (240-260 mg/dL). The major forms of lipoprotein cholesterol routinely measured for clinical evaluations are high-density lipoprotein cholesterol (HDL-cholesterol) and low density lipoprotein cholesterol (LDL- cholesterol). The approaches in both cases involve the selective exposure of the relevant type of cholesterol to the esterase/oxidase/chromogen reactions. This strategy involves the removal of the undesired or “irrelevant” form of cholesterol from the final, color-forming reaction. For instance, if the purpose of the assay is to measure LDL-cholesterol, it is possible to precipitate or eliminate the non-LDL forms of cholesterol (e.g. HDL-cholesterol) and make them inaccessible to the final color forming reaction which would only measure the cholesterol present in LDL.

HDL cholesterol measurements on a specimen from a fasting patient should meet the interim analytical performance goals of total error of 22%, accuracy of ± 10%, and CV of 6% at concentrations of 1.09 mmol/L (42 mg/dL), and SD 0.06 mmol/L (2.5 mg/dL) at concentrations below 1.09 mmol/L (42 mg/dL). The HDL cholesterol reference three-step method is 1) ultracentrifugation of d 1.006g/mL to remove triglyceride-rich lipoproteins, 2) precipitation of apo B-containing lipoproteins from the ultracentrifugal infranatant with heparin and $MnCl_2$, and 3) Measurement of cholesterol in the heparin - $MnCl_2$ supernate by the total cholesterol reference method. Assigned HDL-cholesterol values for calibration and reference materials should be traceable to the reference method for HDL cholesterol. We measured HDL concentration by PEG. The addition of PEG solutions of different concentration and pH results in the selective precipitation of the different lipoprotein fractions. Reagent A is precipitating only the VLDL and LDL fractions, while Reagent B precipitates along with VLDL and LDL, also the HDL2 subclass.

The content of HDL2 cholesterol is obtained calculating the difference between total HDL and the HDL3 subclass.

For LDL cholesterol measurements of a patient specimen, the total error should be of 12%, accuracy should be of ± 4% and CV should be of 4%. For routine analysis (until direct methods are established), analysts should use the Friedewald equation to estimate LDL cholesterol levels from direct measurements of total cholesterol, triglycerides, and HDL cholesterol in specimens from fasting patients. The reference method for LDL cholesterol shall be the ultracentrifugal removal of VLDL, separation of LDL cholesterol by polyanion-divalent cation-reagent, and analysis if HDL cholesterol by the total cholesterol reference method. The LDL cholesterol level is calculated by subtracting the HDL cholesterol concentration from the cholesterol level of the ultracentrifuge d 1.006 g /mL infranate. New routine methods should be validated against a designated reference method. The currently available direct LDL cholesterol methods should be used only for freshly collected serum specimens. We measured LDL cholesterol by heparin precipitation. The low density lipoproteins (LDL) can precipitate by heparin. After precipitation and centrifugation high density lipoproteins (HDL) and very low density lipoproteins (VLDL) remain in the supernatant and can be determined by enzymatic methods.

LDL cholesterol = Total cholesterol — cholesterol in supernatant.

Triglyceride measurements made on specimens from fasting patients should meet the analytical performance goal of total error of 5%, accuracy of ± 5%, and CV of 5%. Instrument and reagent suppliers should provide analytical systems in which glycerol banking can be incorporated into all triglyceride assays. Any specimens with a triglyceride concentrations > 2.83 mmol/L (250 mg/dL) should be glycerol blanked. Specimens can be stored at 4^{o}C up to 3 days; at -20^{o}C up to several weeks; and at -70^{o}C for longer periods.We measured triglycerides by Sigma enzymatic KIT.

4.2. Ultracentrifugation

4.2.1. Preparative density gradient ultracentrifugation

The preparative isolation of serum lipoproteins was carried out by the single-spin density-gradient ultracentrifugation technique. The preparative isolation of serum lipoproteins was carried out in a fixed angle rotor of 20? on a type of 3180 ultracentrifuge made by the Hungarian Optical Works (Budapest).The conditions of the gradient formation and lipoprotein separation were as follows: 3 mL of sera was adjusted to a density of 1.3 g/mL with solid KBr (0.49g/mL) and placed in the bottom of a cellulose acetate centrifuge tube and overlayered with 8.5 mL of 1.00 6 g/mL NaCl solution containing 0.01 g / l EDTA salt made to a volume of 11.5 mL. (In some cases 60?l of 1 mg/mL solution of Sudan Black in ethylene glycol was added to aid in visualisation of the lipoprotein bands. The Sudan Black solution was prepared using a method described by Terpstra.) Tubes were loaded into a P 50 angular rotor of 20 ? for 130 minutes at 50.000 RPM. In the rotor the g force was at the top of the tube g=95030 and at the bottom of the tube it was g=187265. At the end of the run the tubes were removed according to the localisation of lipoprotein bands: 1,5 mL, 4 mL, 2,5 mL 1,5 mL, 2 mL, I. G. free serum protein, HDL, LDL, IDL and VLDL respectively and from these aliquots were made analytical ultracentrifugation runs and radioactive labelling of lipoproteins.

4.2.2. Single-spin density-gradient ultracentrifugation using Schlieren refractometry

We have developed new analytical ultracentrifugal micromethods for the determination of serum lipoproteins directly from ultracentrifugal Schlieren scans. We have used special designed cells and special written software for the analysis of this type of single-spin density-gradient ultracentrifugation**.**

The runs were carried out in an A 65-2 analytical rotor with a type 3180 ultracentrifuge made by the Hungarian Optical Works (Budapest) operating in refractometric Schlieren mode at 546 nm with a high-pressure mercury vapour light source lamp (HBO2000). We applied band-forming, capillary under-layering type single sector centrepieces furnished with special holes. The 4 ° band-forming and 12-mm optical path length centrepieces were assembled with negative angle wedge windows (-1°40') on the bottom of the cell. The setting of the optical system was selected at a constant Philpot angle of 20°. Solid KBr was used to adjust the density of sera or other preisolated lipoprotein fractions (obtained by previous preparative ultracentrifugation isolation step or by precipitation). The samples were adjusted to a density of 1.3 g/mL (0.49 g KBr/mL serum) and 10-100 ?l volumes were injected into the holes of the centrepiece from the upper window with a micro-syringe. The cell was assembled and 0.5 mL NaCl (?=1.006)g /mL solution containing 0.1 g/L EDTA was put into the sector of the centrepiece. Runs were performed at 50.000 RPM and 20 ^{o} C. When the rotor was accelerated to 2000-4000 RPM; the increasing hydrostatic pressure forced the adjusted-density sample from the holes of the centrepiece through capillaries under the physiological solution in the sector. The maximum speed was obtained after 5 min. Photographs of the Schlieren pattern were taken at 10 min intervals from the time that the gradient curves were well visible i.e. at 60, 70, 80, 90 and 100 minutes after reaching full speed. The decomposition of the integrated curve was carried out using the BMDP non-linear regression program, followed by deconvolution algorithm analysis in order to determine the parameters of the Gaussian subclasses. A personal computer, computer graphics and a procedure for minimisation of sums of non-linear squares were applied as follows. The coordinates of the Schlieren distribution curves of samples were obtained from the photograph, input into the computer, interpolated using piecewise cubic polynomials and displayed. The interpolation of the Schlieren distribution curves of gradient baseline was found in the same way, the curves were subtracted and the result was integrated exactly for the ordinates of all pixels. Then, this integral curve was displayed on the screen and one Gaussian curve was adapted to its main peak, guessing

and inputting the three parameters of the Gaussian (mean value, standard deviation, height of maximum). Next, the minimisation procedure was invoked to improve the guessed parameters. During this computation, only the ordinates of the pixels near the main peak were taken into account. The integral curve was displayed once more, now together with the optimised Gaussian. At the place of greatest difference, a new Gaussian was added, and was adapted by hand. Then, the height of the first Gaussian was reduced somewhat and the optimisation procedure was invoked again, but now the ordinates of all pixels (or of every third pixel) on the x-axis were included in the optimization. Only a short part of the axis beginning with x?0 was excluded , since the integral curve starts with zero at x?0 and it makes no sense to try to approximate this by a sum of Gaussian curves. In this way the number of Gaussian curves in the sum was raised step by step until the average deviation between the two curves dropped below 0.25. This is about the error with which values of the original curves can be read from the Schlieren photographs. For the success of the method all three parts of our approach (the stepwise addition of the Gaussian curves, the hand adaptation of the Gaussian curves, and the reduction of the heights of the old Gaussians) were important. The optimisation procedure is specialized to minimise the sums of squares, i. e. in our case

$$F(?_1, ?_1, h_1, \ldots, ?_N, ?_N, h_N) ? ? _{(i?1,N)} ? y(x_i)-h_i \exp(-(x-a_i)^{2/4} ?_i?^2$$

Where h_i is the max. value of function, a_i is the place of the max. value, $2\sqrt{b_i}$ is the deviation.

Here the summation is taken over all x values (ordinates of pixels on the screen) and y? y (x) is the integral curve. The optimisation procedure employes gradient vectors (obtained by a finite difference formula) and Gaussian-Newtonian vectors, which are used to define a plane on which the fuctional F is minimized locally. The minimum value of F is called the fitting error. The LDL

particle concentrations were calculated from the area under the integral of the Gaussian curve using a calibration data constant and from these LDL cholesterol values were derived.

Besides Schlieren optics, ultraviolet absorption optics were used at 254 nm to record the flotation of the LDL band in the cell at the same time, and calibration runs were made with 10-100 ?l sera. The measurement of absorbance allows increased sensitivity and high reproducibility. With the absorption optics, the absolute concentration is available at any point rather than a concentration difference with respect to a reference point. Absorption optics are particularly sensitive for detection of macromolecules containing strong chromophores and the strong lamp output at 254 nm can be characterized lipoproteins with good signal to noise ratio at concentrations as low as 10 µg/mL.

4.2.3. Investigation of specific lipoproteins fractions:

LDL investigation method

2x 50 ?l LDL aliquots volumes were injected into the holes of the centrepiece from the upper window with a micro-syringe. The cell was assembled and 0.5 mL NaCl (?=1.042) g/mL solution containing 0.1 g/L EDTA was put into the sector of the centrepiece. Runs were performed at 50.000 RPM and 20 ^{o} C. When the rotor was accelerated to 2000-4000 RPM; the increasing hydrostatic pressure forced the adjusted-density sample from the holes of the centrepiece through capillaries under the salt solution in the sector. The maximum speed was obtained after 5 min. Photographs of the Schlieren pattern were taken at 20 min after reaching full speed.

HDL investigation method

2x 50 ?l sera or isolated HDL aliquots fractions were adjusted to a density of 1.3 g/mL density with solid KBr and were injected into the holes of the centrepiece from the upper window with a micro-syringe. The cell was assembled and 0.5 mL NaCl (?=1.042)g /mL solution containing 0.1 g/L EDTA. Runs were performed at 50000 RPM and 20^{o}C. Photographs were taken after reaching full speed at 80 min.

VLDL investigation method

2x 50 ?l isolated VLDL aliquots were injected into the holes of the centrepiece from the upper window with a micro-syringe. The cell was assembled and 0.5 mL NaCl (?=1.006)g /mL solution containing 0.1 g/L EDTA. Runs were performed at 50000 RPM and 20 ^{o} C . Photographs were taken after reaching full speed at 20 min.

IDL investigation method

2x 50 ?l isolated IDL aliquots fractions were adjusted to a density of 1.063 g/mL density with solid KBr and were injected into the holes of the centrepiece from the upper window with a micro-syringe. The cell was assembled and 0.5 mL NaCl (?=1.006)g /mL solution containing 0.1 g/L EDTA. Runs were performed at 50000 RPM and 20 ^{o} C. Photographs were taken after reaching full speed at 20 min.

4.2.4. Isolation of lipoprotein fragments for analytical ultracentrifugation

The components of aorta were extracted in physiological solution after homogenisation and centrifuged of the cells for 30 minutes. The extracted components were investigated in the supernatant fraction by analytical ultracentrifugation

Modified forms of LDL, especially with characteristic of LDL- have been isolated from human plasma and from the plasma of animals which have had experimentally-induced atherosclerosis.

4.2.5. Combination of precipitation and analytical ultracentrifugation methods

To a volume of serum (1mL) 0.1 volume of the Heparin manganese reagent solution was added (Heparin sodium 2500 units/mL).This resulted in a final concentration of 4.1 mg/mL for Heparin and 0.083 M MnCl2. After 10- 15 min. at room temperature the samples were centrifuged for 30 min at 3000 RPM and 20?C. The supernatant was completely removed and any resultant precipitate was dissolved or suspended in 0.2 mL volume of another aliquot of the same sera. The density of the aliquot was adjusted with solid KBr according to analytical ultra-centrifugation method. Aliquots of supernatant and infranatant serum were taken for Schlieren analysis after adjusting the density of the solutions with solid KBr to 1.30 g/L and underlayering by physiological solution, and the lipoprotein distribution was investigated using Schlieren optics. The divergences in the Schlieren distribution profiles of the lipoproteins were used to follow the interaction of LDL and precipitation reagents as heparine, heparane, chondroitin 4-6 sulphate and anti-apo-B, anti-apo-A1 and anti apo A2 immunoprecipitating reagents.

4.3. Oxidation of Low Density Lipoprotein

OxLDL were obtained by copper oxidation: LDL in 0.15 M NaCl were oxidized by incubation for 30 min at 37 °C with copper sulfate (2 µM). The oxidation of LDL was carried out by Cu++ ions and increased by gamma radiation. Doses of gamma irradiation were from 1 Gray to 10 Gray.The extent of oxidation was measured by spetrophotometry on he basis of absorbance values. Oxidation was stopped by adding EDTA (2 µM) to the solution.

4.4. Lipoprotein labelling with 99mTc

LDL were labeled with 99mTc according to Lees et al.,(1985). Briefly, LDL (4-8 mg protein) was mixed with 40-60 mCi 99mTc-pertechnetate (TcO_4^-) and 10 mg sodium dithionite, which was dissolved just before use in 0.5 M glycine buffer, pH 9.8, and the mixture was incubated for 30 min. Radiochemical purity and in vitro stability were controlled by paper chromatography in aceton. 99mTc-LDL was separated from free 99mTc by Sephadex G25 chromatography. The purified 99mTc- LDL was dialyzed against 150 mM NaCl, sterilized through a 0.22-µm filter. The labelled lipoproteins were used for experiments immediately. 99mTc-LDL was separated from free 99mTc by Sephadex G25 chromatography. The purified 99mTc- LDL was dialyzed against 150 mM NaCl, 1 mM EDTA, pH 7.4, at 4 ^{o} C for 14 h, sterilized through a 0.22-µm filter. Protein content was determined in an assay of dye-protein complex with Comassie Brilliant Blue.

4.5. Histological examination of Arteries

The exsanguinated rabbits were autopsied. The whole length of aorta and carotid arteries were isolated. Segments of the aorta and carotis containing atherosclerotic lesions were fixated in formaldehyde (10%) and embedded in paraffin. Serial 8- to 10-µm-thick sections were stained for lipid by using hematoxylin-eosin and oil-red o.

4.6. Investigation of the accumulation of low- density lipoproteins in mononuclear cells

Fresh rabbit blood was diluted (3:1) with PBS (150 mmol/L NaCl, 8.5 mmol/L Na_2HPO_4, 1.5 mmol/L NaH_2PO_4, pH 7.4) containing 200 U/mL of heparin. Of this solution, 15 mL was brought on 12 mL of Ficoll-Paque density gradient (d=1.077), and centrifuged for 45 minutes at $750g_{av}$ at room temperature. The mononuclear interphase was collected, washed 3 times with cold PBS/heparin, and resuspended in RPMI 1640 medium containing penicillin/streptomycin (100 µg/mL), L-glutamine (2 mmol/L), supplemented with 10% BCS. After overnight incubation at 37°C in the incubator (5% CO_2, 95% air), nonadherent cells were removed by aspiration followed by washing with RPMI 1640.

We compared the LDL uptake of normal and pathological mononuclear cells with the analysis of the supernatant before and after incubation with LDL fraction with the analysis of the supernatant before and after incubation with LDL fraction isolated from sera or atheroma.

4.7. Imaging studies

After overnight fasting, rabbits were anaesthetized by 10 mg/kg of ketamine (SBH-Ketamin, SBH, Hungary) and 5 mg/kg of xylazine (Primazin, Alfasan, The Netherlands), given intramuscularly. A 20G-cannula (Medicor, Hungary) was placed into the marginal ear vein and approximately 500 MBq of the labelled 99mTc-LDL was injected into the animals. Images were acquired with a large field-of-view gamma camera (Nucline X-Ring, Mediso, Hungary), with a low-energy high-resolution collimator. Dynamic studies were run in either 30-seconds or 1 minute frames for 15 minutes immediately after injection in left lateral view. Static images were acquired 30 minutes, 1h, 2h, 4h and 24h post injection at a 256x256x8 matrix size until 600000 counts were reached. For each time point, ventrodorsal, left lateral zoom and ventrodorsal view whole body static images were taken. Images were processed with a dedicated software, InterView 1.83.2 (Mediso, Hungary).

4.8. Animals

All care was provided for the animals according to national and international standards, and the experimental protocol was accepted by the local Animal Experimental Ethic Committee.

4.8.1. Wistar rats

Wistar rats (LATI, Hungary) (180g to 200g)(n= 20) were maintained on a stock powder diet and with water *ad libitum*. Suckling rats were removed from the mothers on 14, 21 and 28 day during short treatment.

In each case, sera were derived from pools made up of samples from 5 animals. The blood samples were taken from 14-day, 21-day and 28-day old suckling rats after removal from the mother and 3 month old rats, sera after fasting to14 h, the animal were bled from a vein under light anesthesia. In the Triton treatment, the rats received an i.p. injection of Triton WR 1339 (a 7% solution in normal saline) at 350 mg/kg. In the Triton treatment group, the rats were then bled from an orbital vein about 20 h after Triton administration.

4.8.2. Atherosclerotic rabbit model

New Zealand White rabbits (LATI, Hungary) weighing 3 to 3.5 kg were housed in separate cages at room temperature and submitted to a 12 h light /dark cycle. Rabbit chow and water were available ad libitum to both rabbit groups. The rabbits were fed on a diet of normal rabbit chow (Biofarm, Hungary) enriched with cholesterol (Sigma) to a content of 1 and 2% for sixty days. The serum cholesterol and triglycerides levels of the NC and HC rabbits were determined by enzymatic methods at the beginning of the experiment (normocholesterolaemia), during feeding and after sixty days (hypercholesterolaemia).

4.8.3. Tumor-bearing nude mice

Nude mice(LATI, Hungary) were used for the experiments.

Traditional approaches of monitoring cancer growth in mouse models have largely used human implanted tumours. A variety of non-invasive high-resolution imaging methods are now available for the detection and monitoring of deep-seated cancers, as well as their metastases, in mouse models. In the simplest image-acquisition mode, a ‘picture’ is acquired

The clinical needs for more specific molecular imaging tools are obvious.

Tumor cell lines. Human cancer cell lines SPC-A1 adenocarcinoma of lung cancer, Bcap-37 Breast cancer, were obtained from Cancer Institut.

Tumor cell implantation. Tumor cells were transplanted into mice. Approximately 10^6 cells of each cell line were injected subcutaneously into a right flank of mouse.

Scintigraphy. After 4~5 weeks of tumor cell implantation or when the tumor had reached a diameter of = 1cm, the mice were anaesthetized with an intramuscular injection of ketamine (80 mg/kg) and an intraperitoneal injection of thiopental sodium (0.625mg/kg) before imaging. The scans of Tc-99m LDL scintigraphy with an acquisition counts of 500k-800k, in a 512×512 matrix, zoom 2.67 and took mice placed on surface of the detector were performed at 1-6 hours after tail vein injection of 74MBq in 0.05mL every tumor-bearing mouse using a double headed gamma camera equipped with a low-energy high-resolution parallel-hole collimator.

5. RESULTS

The preparative and analytical ultracentrifugal analysis, which are based on physical properties permit the investigation of lipoproteins in natural state. Preparative density gradient ultracentrifugation allows the simultaneous isolation of major lipoprotein density classes ie. VLDL, IDL, LDL, HDL forming discrete bands in the preparative tubes.

In order to verify the lipoprotein assignment we have examined the lipoprotein samples both obtained from normal subjects and patients with different types of HLP. Analyses for the principal lipid constituents was done on the whole serum and the isolated lipoprotein fractions.The bands of the migrated lipoprotein were constant or only very small differences were noted. The localisation of lipoprotein bands in the tubes indicated that three fractions at the bottom correspond to free serum protein, lipoproteins bands with properties of HDL were present in the gradient fractions from 4 to 10, band with the properties of LDL were present in fractions of 12 to 17, in fractions of 18 to 19 were present in type III HLP samples IDL, while band at the top of the tube isolated in fractions of 20 to 23 corresponded to VLDL.

VLDL, LDL, HDL cholesterol contents were determined and looking at lipoprotein fractions pertaining to the total cholesterol. These results indicated that the density gradient ultracentrifugation in the recoveries of cholesterol according to the density classes of lipoproteins was acceptable accurate, because he cholesterol content of the various fractions related to the original serum was within 10 % . We determined VLDL, LDL, HDL cholesterol in normal sera and in sera of patients of type II B, type III, type IV, type V HLP. Distribution of cholesterol were taken from the tubes in the numbers of 23 fractions after single spin density centrifugation.

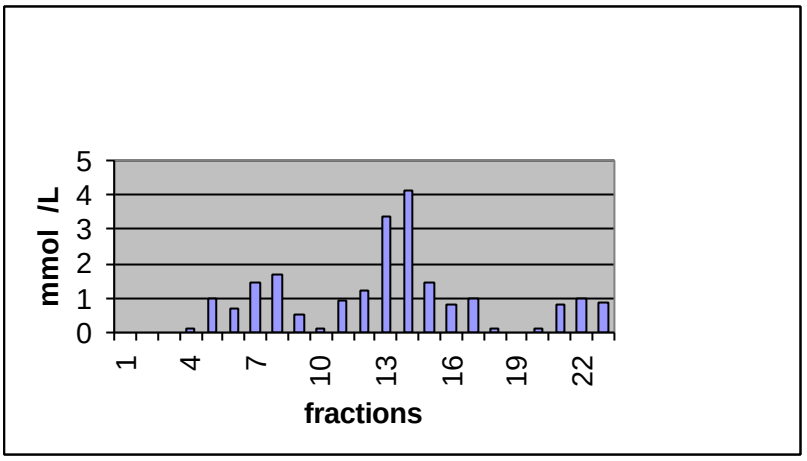
In the normolipidemic sample 210-220 mg/dL (5.42-5.68 mmol/L) was the upper limit for serum cholesterol, VLDL cholesterol contained less than 40 mg/dL (1.03 mmol /L, LDL cholesterol was up to 160 mg/dL (4.13 mmol/L) and HDL cholesterol was in the range up to 60 mg/mL (1.55 mmol/L).(in Fig 1A) In the type II serum LDL cholesterol was greater than 200 mg/dL (5.16 mmol/L) (in Fig1B). In the type III, type IV, type V VLDL cholesterol was isolated greater than 9O mg/mL (2.32 mmol/L).

Distribution of triglycerides were taken also from the tubes in the numbers of 23 fractions after single spin density centrifugation.

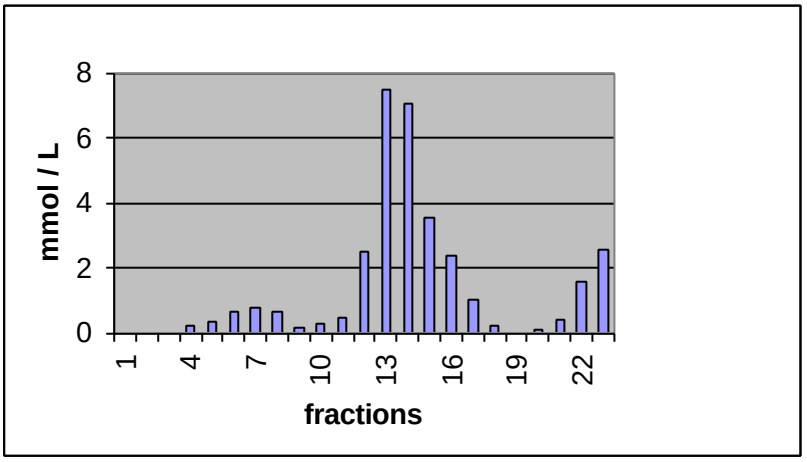
Triglyceride isolated from normolipidemic sera containing less than 100 mg/mL (1.14 mmol/L) VLDL triglyceride. Triglyceride was measured in subject with mildly elevated VLDL triglyceride 120 mg/mL (1.37 mmol/L) and in subject with severe hypertriglyceridemia greater than 250 mg/mL (2.85 mmol/L) VLDL triglyceride.

Prestained normal serum contained a trace of VLDL at the top of the tube and an intensely stained band containing LDL at the middle of the tube and a faintly stained band of HDL at the lower region of the tube. Patients with type II HLP had an intensely stained board band (LDL) at the middle of the tubes. Prestained samples from patients of type III HLP revealed a diffusely stained band between VLDL and LDL due to the presence of floating IDL (intermediate

density lipoprotein).In serum from patients with type IV and type V an intensely stained band at the top of the tube



Figur 1.A Cholesterol distribution in normal sample



Figur 1.B Cholesterol distribution in type II HLP sample

We haves tudied cholesterol levels from healthy subjects? sera (N= 20) and one from CHD patients?sera (N= 30).

The characteristics of the population studied are given in Table 1.

Table 1 Characteristics of the population studied

	Normal subjects (N = 20) (men=12, women=8)
Age, (years)	41 ? 8
Serum cholesterol	4.17 mmol / l ?0.72
Serum triglycerides	1.29 mmol / l ?0.81
High-density lipoprotein Cholesterol	1.06 mmol / l ?0.32

	Coronary heart disease patients (N = 30) (men=24, women=6)
Age, (years)	48 ?12
Serum cholesterol	4.56 mmol / l ?0.92
Serum triglycerides	2.72 mmol / l ?0.98
High-density lipoprotein cholesterol	0.86 mmol / l ?0.35

Analytic ultracentrifugation provides separation of particles based on density and Svedberg flotation intervals. LDL is not a homogeneous category of lipoproteins but consists of a set of discrete subspecies with distinct molecular properties, including size and density. In most healthy people, the major subspecies are large or buoyant, whereas the smaller denser LDL subspecies are generally present in small amounts. We obtained quantitative results by measuring the Schlieren diagrams between the sample curves and the reference baseline curve by using computerised numerical and graphic techniques. The lipoprotein particle concentrations were calculated from the area under the integral of the Gaussian curve using calibration data constant.

Using serum pool from healthy subjects? sera (N= 20) we studied LDL heterogeneity.

The Schlieren patterns obtained for serum pools from healthy subjects and from CHD patients are also shown in Fig. 2 A and graphical presentation of the evaluation of a Schlieren curve obtained for serum pool from healthy subjects is shown in Fig.2 B)

Fig. 2 A
Schlieren diagrams from healthy subjects

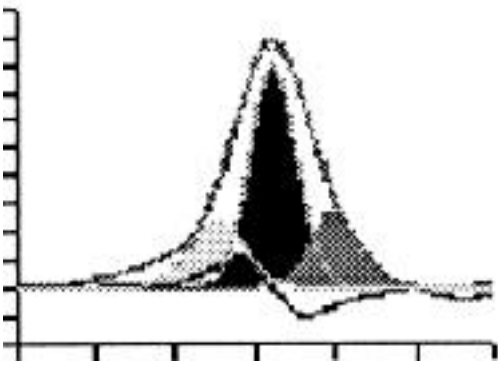


Fig. 2 B
On the basis of integration and deconvolution we have obtained three subfractons of LDL

Ultraviolet absorption optics was used at 254 nm to record the flotation of the LDL band in the cell at the same time, as the recording of Schlieren diagrams in Fig.3.

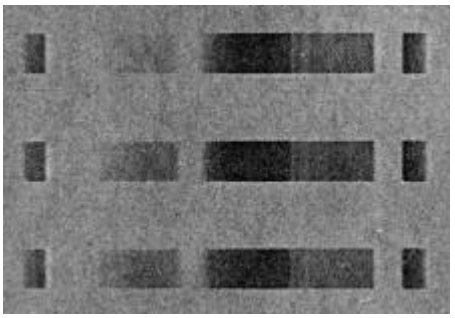


Fig.3 Flotation of LDL band by ultraviolet absorption optics

In the middle of the cell can be seen LDL flotation band by ultraviolet absorption optics.

The photographs of ultraviolet absorption patterns of LDL were taken at 60, 70, 80 min after the attainment of full speed (50,000 rpm).

With the absorption optics, the absolute concentration is available at any point rather, than a concentration difference with respect to a reference point. The LDL cholesterol concentration of this sample was130 mg/dL.

Schlieren scans of coronary heart disease patients show predominatelly assymetrical peak, where the mass of the lipoproteins is distributed over a broader density region. The small LDL is linked to atherogenicity and are able to infiltrate the arterial wall faster than large LDL particles, these particles are more susceptible to oxidative damage.

A multiple peak or a polydisperse LDL pattern within the Schlieren samples curve of LDL was more often found in samples from CHD patients than in controls are shown in Fig 4 A,B,C.

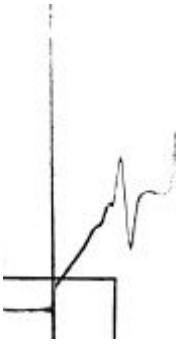


Fig.4A ***Fig.4B*** ***Fig.4C***

Schlieren samples curve of LDL

We determined the qualitative and quantitative characteristics of lipoprotein particles in patients defined as presenting type II and type IV hyper-lipoproteinemia (Fig 5 A, B).

Fig 5A
Type II HLP

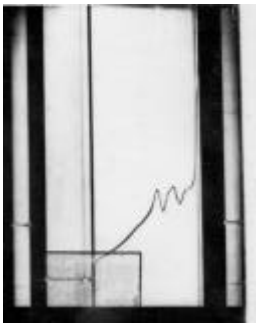


Fig 5 B
Type IV HLP

The photograph of the Schlieren pattern was taken 80 min after attainment of full speed (50,000 RPM).

Schlieren diagram was produced of LDL in type II HLP sera containing 205 mg/dL LDL cholesterol. Schlieren diagram was produced of LDL in type IV HLP sera containing 80 mg/dL LDL cholesterol.

Schlieren detection of concentration changes of LDL was established by heparin precipitation

LDL precipitation with heparin can be seen in Fig.6.

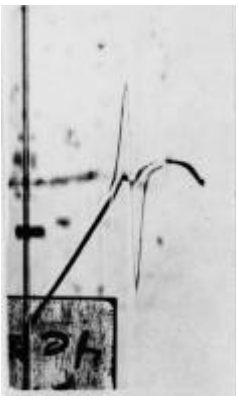


Fig. 6 LDL precipitation with heparin

The photograph of the Schlieren pattern was taken at 80 min after attainment full speed (50,000 rpm).

Before heparin precipitation, the LDL cholesterol concentration was about 130 mg/dL, after heparin precipitation (not full precipitation) about 15 mg/dL remained in the serum aliquot (in two cells run at the same time). Similarly to heparin, the interaction of LDL with other molecules (e. g. Glycosaminoglycan or Lipoprotein Lipase) can be used before the radioactive labeling of lipoprotein aliquots to increase modified LDL uptake of endothelial cells for scintigraphic detection in animal models or in the *in vitro* models.

Normal serum with 65 mg/dL HDL cholesterol level from whole serum together with LDL peak can be seen in Fig.7.

Fig.7 HDL cholesterol level from whole serum together with LDL peak

The presentation of lipoprotein Schlieren data to gain insight of the profile of LDL and HDL concentrations.

We have determined total, VLDL, LDL, HDL cholesterol levels in rat sera pools of 2-, 3-, 4-weeks and 3-months age rat samples. The great differences in the cholesterol values were between 2 and 3 weeks old rat sera pools according to total cholesterol and LDL cholesterol levels. Cholesterol levels of rat samples are given in Table 2.

Table 2. Cholesterol levels of the rat sample pools studied

Rat age	Number of samples	Total serum cholesterol (mg/dL)	VLDL cholesterol (mg/dL)	LDL Cholesterol (mg/dL)	HDL cholesterol (mg/dL)
2 weeks	5	298.3	38.1	202.5	57.7
3 weeks	5	121.8	18.2	42.2	61.4
4 weeks	5	108.6	10.6	38.9	59.1
3 months	5	85.5	6.6	15.6	63.3

By means of analytical density gradient it can be seen the characteristic lipoprotein profile of the various lipoprotein fraction of animal species and such data may be useful in selecting of suitable animal models.

Schlieren diagrams of 2, 3, week old rat sera pools are shown in Fig.8 A,B.

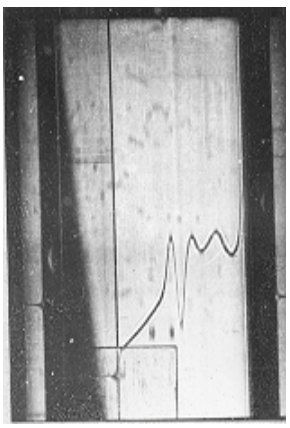


Fig. 8A

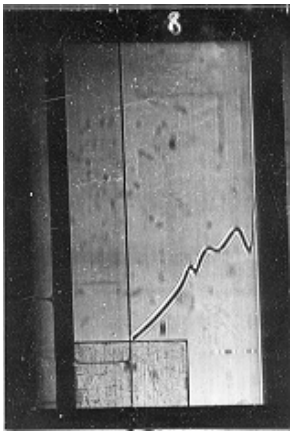


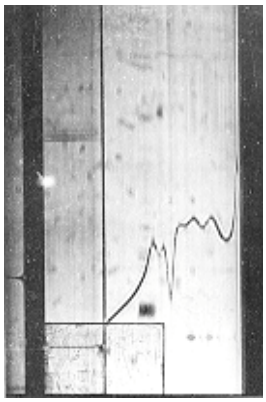
Fig 8 B

Schlieren diagrams of 2, 3, week old rat sera pools

The most characteristic changes in the lipoprotein distribution profiles analysed on the Gaussian curves was between two and three week old rat samples. Low Density Lipoprotein (LDL) (more than 200 mg/dL cholesterol) in two week old rat sera pool have decreased in three weeks rat sera pool (less than 50 mg /dL cholesterol).

The effect of Triton on plasma LDL concentration are shown on the basis of Schlieren pictures. We have detected the decrease of LDL concentration values in all rat sera pools. In the early phase of the run up to 20 min we have detected increased level of VLDL in all sample pools. The effects of the Triton can be observed on the same age animal samples. The integrated Schlieren curves indicated changes in the LDL concentration. Screening of the LDL by analytical ultracentrifugation demonstrated that the LDL content decreased by more than 50%.

Schlieren diagrams of 2, 3 week-old rat serum pools (A and B respectively) 20 h after Triton administration are shown in Fig. 9 A,B.



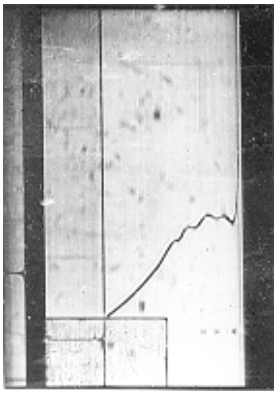


Fig. 9 A ***Fig. 9 B***

Schlieren diagrams of 2, 3 week-old rat serum pools

Schlieren diagrams of rat serum pools at 80 min after attainment of full speed (50.000 rpm).

We have designed in this experimental order to detect LDL receptor or scavenger receptor uptake of low density lipoproteins (without labelled lipoproteins) in the native state of serum after the incubation of serum with mononuclear cells by Schlieren optics.

We have created, using eight well known herbs (official monographs of the Hungarian Pharmacopoeia), which has got antihyperlipidemic, antioxidats effects. The plant constituents (Urticae folium, Calendula officinalis, Tilae flos, Sambuci flos, Hyperici herba, Equiseti herba, Achilleae millefolium,Veronica officinalis) were used for the tea blends. The compounds were chemically analysed and found to contain, a high concentration of flavonoids including quercetin, morin, gossypetin, chrysin, myricetin, rutin, catechin and its derivatives, and the oligomeric proanthocyanidins and mineral substances. After only four weeks consumption of the herbal compound, in tee form, significant reductions of serum triglycerides, total cholesterol and LDL cholesterol were produced. The herbal constituents which were extracted by aqueous decoction were lyophilised and its free radical scavenger and antioxidant effect was investigated on the basis of superoxide dismutase activity. Plants have showed high SOD activity with free radical scavenger effects and high antioxidant effects. The degree of antiradical effect was more than 50 per cent of the herbal blend.

Clinical tests verified serum cholesterol and HDL cholesterol lowering and HDL cholesterol increasing effect of the plant extract. Oxidation of LDL was used as a model of the anti-lipid peroxidation activity of flavonoids. The mechanism by which flavonoids inhibit LDL is not totally known, but it is thought that they reduce free radical formation, protect LDL- tocopherol or regenerate oxidized LDL-tocopherol, and/or sequester metal ions which participate in oxidation reactions. The antioxidative activity of polyphenol compounds typically present in herbal compound. A number of naturally occuring antioxidant compounds

have been found to strengthen the resistance of LDL to oxidative modification *in vitro.* Our interest has been focused on plants which are known to contain high content of polyphenol compounds. With these in consideration, our objectives were to determine the effects of extract of herbal on $CuSO_4$ –mediated LDL oxidation and to establish whether the extract could terminate LDL oxidation once initiated. The Cu^{2+} -initiated oxidation of LDL was significantly inhibited in a concentration-dependent manner by adding flavonoids directly to the LDL oxidation mixture. The lipophilic antioxidants carried in LDL could protect it against oxidation. Enrichment of bioactive substances in plasma lipoproteins such as isoflavones and other antioxidants could be achieved by diet supplementation. In vitro testing of the antioxidant activity of the antioxidant substance in the extract, was found to be stronger as an antioxidant than vitamin E.

The rabbit is an important model for the study of the relationship between plasma cholesterol metabolism and atherosclerosis. White rabbit cholesterol level is in the range of ~30-65 mg/dL, with young animals (<3 kg body weight). High density lipoproteins (HDL) are the most abundant lipoprotein class in the normal rabbit transport more than half of the circulating cholesterol in fasting rabbit plasma. The rabbit have rapidly developed severe hypercholesterolemia leading to premature atherosclerosis in response to dietary manipulation. The laboratory chow supplemented with 1-2% cholesterol, rapidly leading to plasma cholesterol concentrations that can exceed 2,000 mg/dL, while HDL cholesterol is decreased. Supplementing the diet with cholesterol rapidly results in a marked increase in the production of cholesteryl ester-rich, beta-migrating very low density lipoproteins (beta-VLDL) by the liver and intestine. The VLDL become the major class of plasma lipoproteins. Subsequent clearance of the beta-VLDL by the liver is reduced due to a downregulation of cell-surface lipoprotein receptors and the saturation of the remaining receptors. The beta-VLDL, including chylomicron remnants, that accumulate in the circulation are highly atherogenic. Plasma levels of cholesterol correlate closely with the extent of lesion development.

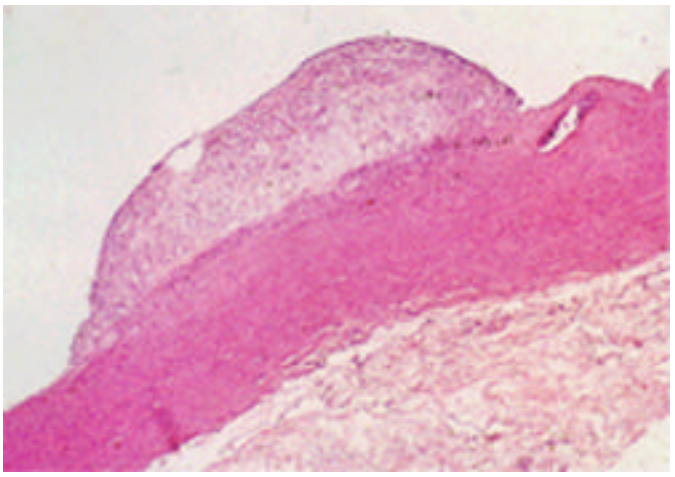


Fig.10 Segment of the aorta containing atherosclerotic lesion

Autopsies revealed the formation of atheromatous plaques on the inner aortic surfaces of rabbits in induced hyperlipiemia.

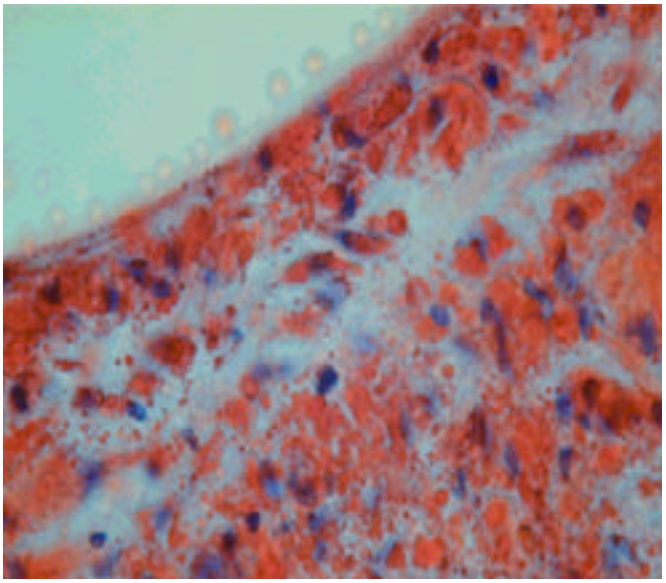


Fig.11 Deposition of lipids in atherosclerotic lesions

Staining of aortic sections for lipid using oil red O revealed that cells in the atherosclerotic lesion contained a high content of lipid.

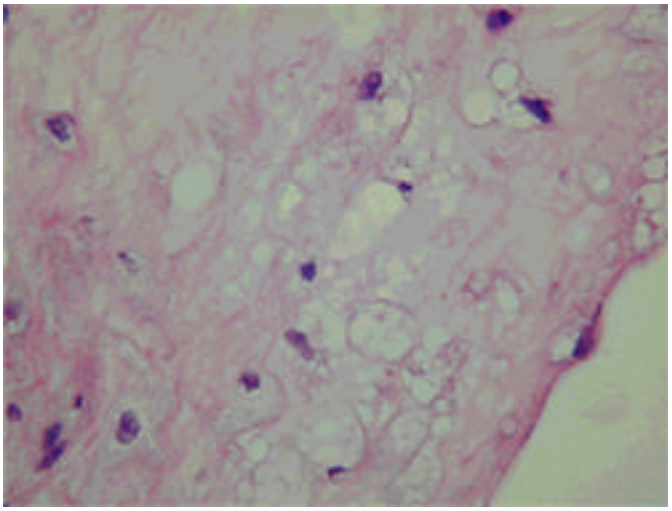


Fig. 12 Atherosclerotic plaque with foam cells

Staining with Haematoxilin –Eosin 600x

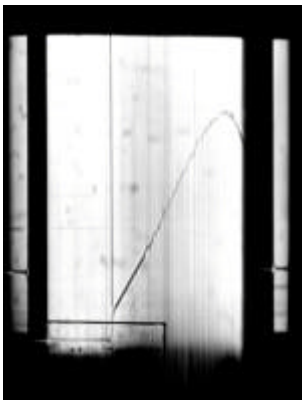


Fig. 13 Analytical ultracentrifugation of lipoprotein fragments isolated from plaque tissue

The photograph of the Schlieren pattern was taken after 80 min when the gradient baseline was well visible after attainment of speed (50,000 rpm). Density gradient ultracentrifugation revealed LDL-like particles in the plaque tissue. This Schlieren picture shows that particles accumulate in the arterial wall, partly in the form of lipoprotein-like particles, contributing to plaque formation.

Gamma camera scintigraphy the detection and quantification of gamma-emitting radionuclides is commonly used to track individual molecules or cells. For diagnostic imaging agents, technetium-99m is a frequently used radionuclide, because it has optimal nuclide properties (half-life of 6h and appropriate Gamma-energy of 140 keV). In vivo detection of atherosclerotic lesion may be a good means to image atherosclerotic plaques using a receptor specific radiopharmaceutical of isolated and prepared modified lipoprotein fractions. Dynamic gamma camera imaging revealed a faster plasma clearance of oxidized –LDL than native-LDL. The rapid blood clearance of oxidized-LDL resulted as a consequence of more rapid uptake by macrophage rich tissue.

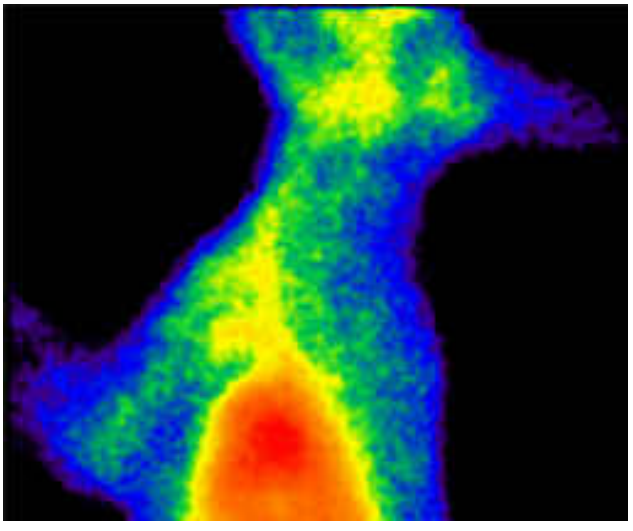


Fig. 14 Gamma camera in vivo scintigraphy of a hypercholesterolemic rabbit

1 h post-injection left lateral view Image of New Zealand White rabbit of 360 MBq ^{99m}Tc-labeled oxidized human LDL .LDL was oxidized by copper and by gamma irradiation of 5 Gray. The rabbits were fed with a diet containing 2% of cholesterol, and plaques were visualized in the trunk pulmonary and common carotid arteries.

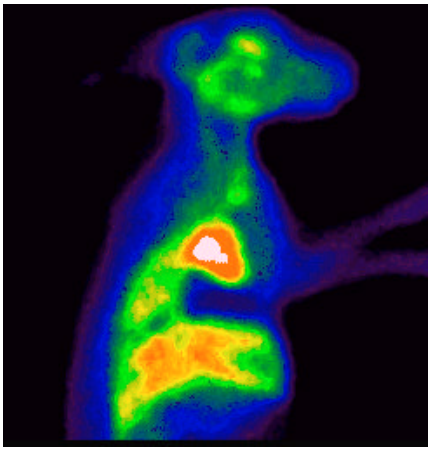


Fig. 15 Gamma camera in vivo scintigraphy ofhypercholesterolemic rabbitThe rabbits were fed with a diet containing 2% of cholesterol. Lateral view of rabbit at 1 h post injection of 10 mCi (370 MBq) 99Tc-LDL. The radiolabelling efficiency was more than 90 %.Uptake of the radiolebelled LDL can be observed in the heart, liver, lungs and caroted arteries .

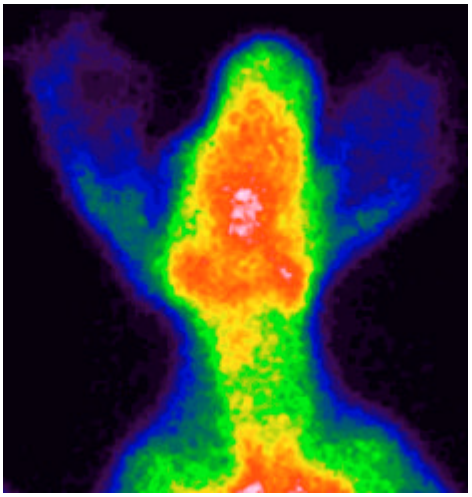


Fig. 16 Gamma camera scintigraphy of hypercholesterolemic rabbit

The rabbits were fed with a diet containing 2% of cholesterol.
Ventrodorsal view of a rabbit at 1h post injection of 10 mCi (370MBq) 99m Tc-LDL. Uptake of the radiolabelled LDL can be observed in the brain, the aortic arch and the common carotid arteries.

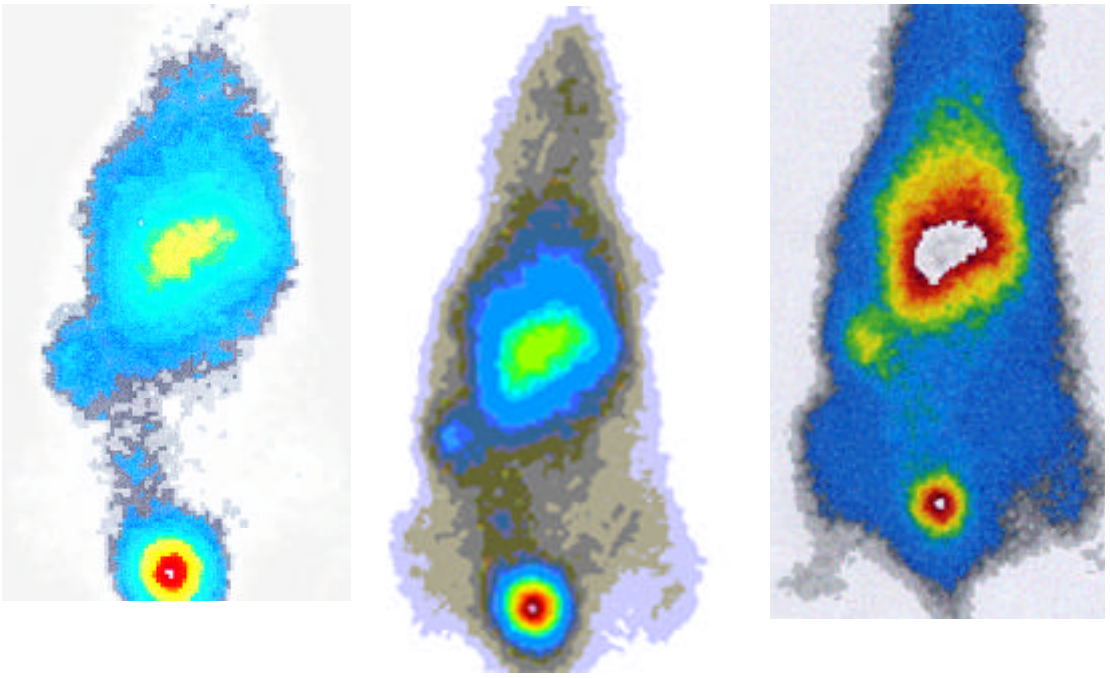


Fig.17 Tumor targeting with 99mTc labelled LDL in nude mice

In vivo tumor imaging after injection of 120 MBq of 99mTclabelled human LDL. Human tumor cells were developed.(Tumors can be seen on the left side of the pictures). Certain tumour cell lines are known to accumulate Low Density Lipoprotein (LDL) several times faster than comparable normal cells.

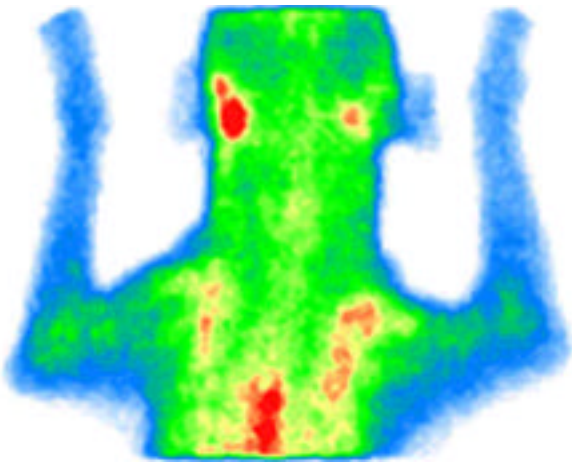


Fig.18 Tumor targeting with 99mTc labelled LDL in dog with osteosarcoma
Scintigraphic detection of osteosarcoma was performed in scapula after injection of 600 MBq of native ^{99m}Tc-labelled human LDL

We calculated the blood residence time from dog blood clearance. The residence time (?, hours) means an effective time that the administered activity spends in the source organ and in our case is 6.13 hr. Pharmacokinetic properties of ^{99m}Tc-LDL in dog blood are seen in Fig. 19.

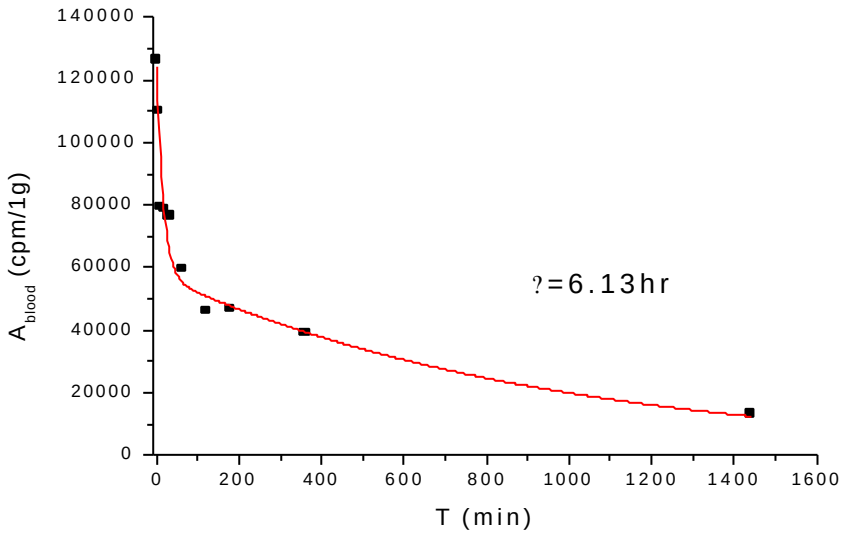


Fig. 19 Pharmacokinetic properties of ^{99m}Tc-LDL in dog blood

The residence time depends on the radioactive decay and the physiologic behaviour of the radiopharmaceutical. Practically the residence times are obtained from the biodistribution data by making the time-activity curves and calculating the cumulated activity (time integral of the activity).

The dosimetry estimates are based on biodistribution (blood clearances) studies using methods developed for radiation dose calculations by the Medical Internal Radiation Dose (MIRD) Committee of the Society of Nuclear Medicine.
The effective dose resulting from i.v. administered activity of 400 MBq is typically 1.41 mSv (per 70 kg individual). Comparative effective dose between nuclear medicine and radiological investigations can be seen in Table 3.

Table 3. Comparative effective dose between nuclear medicine and radiological investigations

X-ray exam	Effective dose (mSv)	Nuclear medicine	Effective dose (mSv)	CT exam	Effective dose (mSv)
Chest (AP)	0.04	Heart (^{99m}Tc-MIBI)	6.66	Chest	7.8
Abdomen	1.2	**^{99m}Tc-LDL**	**1.41**	Abdomen	7.6
Skull	0.1	Brain (^{99m}Tc-HMPAO)	6.88	Head	1.8
Lumbar spine	2.1	Lung (^{99m}Tc-MAA)	1.00		
Thoracic spine	1	Bone (^{99m}Tc-MDP)	4.22		
Pelvis	1.1	Kidneys(^{99m}Tc-DMSA)	1.63	Pelvis	7.1

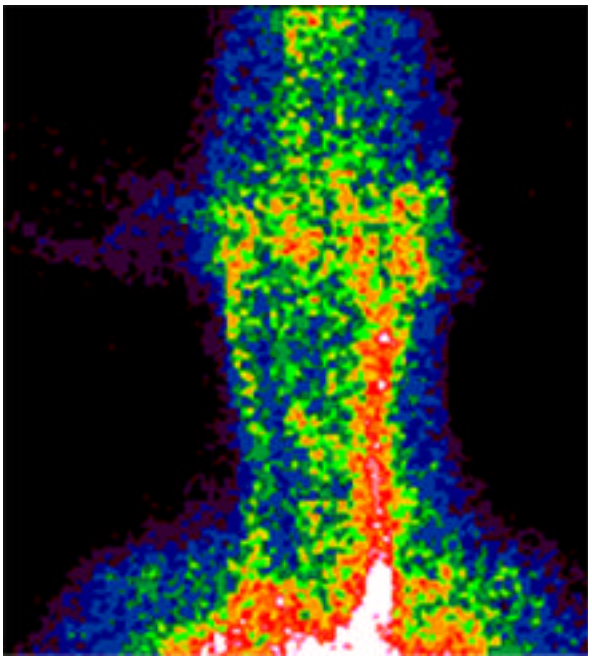


Fig. 20 Scintigraphic imaging of the vascular inflammatory with 99mTc-labelled LDL

Ventrodorsal view of a dog at 1h post injection of 10 mCi (370MBq) Tc-LDL.

Scintigraphic detection of the localization of inflammation of left carotid artery can be seen on this image. Endothelialization injury of dog was made in the left carotid artery using balloon angioplasty.

6. DISCUSSION

LDL are implicated in an atherosclerotic process in which the blood flow is restricted by cholesterol-related plaque in the vessels and becomes a major component of atherosclerotic plaque lesions. 99mTc(Technetium)-labelled lipoproteins can be used as a radiotracer because it acts as an intracellularly trapped ligand providing an scintigraphic measurement of lipoprotein uptake by tissues. We have developed rapid and reproducible ultracentrifugation methods to obtain lipoprotein aliquots for radiolabeling and to evaluate lipoprotein labeling techniques which are suitable for the scintigraphic delineation of

experimental atherosclerotic lesions. We have described a short-run analytical ultra-centrifugation method for quantification of the total LDL content and of its sub-fractions from whole serum within 100 min. No pre-isolation of lipoproteins, a time-consuming step is needed. Because of its rapidity and convenience lipoproteins probably remain fully native. In the analysis of samples the method appeared to be reproducible. Certainly this method is not suitable for routine analysis. Rather it can be regarded as a reference method because the risk of denaturing of the sample is minimal compared to the commonly used methods involving density-gradient ultra-centrifugation or gradient gel electrophoresis methods. In normolipidemic serum three main LDL sub-fractions could usually be identified. According to other researcher, for example, (Swinkels et al.,Fischer et al.) the number of LDL sub-fractions showed a good agreement with our results. The described method proved to be useful for a clear and immediate visual presentation of the concentration values of LDL and for the identification of the heterogeneity of LDL variants without the need for the preparative isolation of that density class. LDL Schlieren curves were more often characterised by a preponderance of less- and more-dense sub-fractions in samples of patients with documented coronary artery disease than in controls. We determined the qualitative and quantitative characteristics of lipoproteins particles in normal subject and in patients defined as presenting type IV and type II hyper-lipoproteinemia. A striking feature of the rat serum data is the monitor of the high concentration of LDL in 2-week-old rat serum pool, the concentration being greatly decreased in 3-weeks rat serum pool. Experiments were performed to determine the changes in the LDL distribution profiles after a simple injection of Triton .The Schlieren curves reveal that there is a decrease in the concentration of LDL. We applied analytical ultracentrifugation to detect the LDL after its isolation by precipitation from the serum and dissolution of the precipitate in another aliquot of the same serum. By a combination of precipitation and analytical ultracentrifugation methods we obtained sufficient quantities of LDL in the 3-months rat serum pool for the analysis of Schlieren curves.

From a screening aspect, we regard the procedure as a very promising technique with which to visualize LDL via Schlieren curves for some of the domestic and laboratory animals frequently used for studies on atherosclerois. It is known that diet-induced hyperlipidemia and atherosclerotic lesions develop more easily in some species than in others. For example, the rabbit develops hyperlipidemia and arterial lesions on less extensively modified diets than do rats. In the analysis of serum samples the method appears reproducible and linearity was observed in the analyses of sample quantities. Modified forms of LDL, especially with characteristics of serum LDL- were detected in atheroma and the plasma of rabbits experimentally-induced atherosclerosis. Histological examination showed the formation of atheromatous plaques on the inner aortic surfaces of rabbits with induced hyperlipidemia.

Combination of preparative and analytical ultracentrifugation was used for the investigation of human low-density lipoprotein aliquots to prepare radioactive-labelled lipoproteins as well as in rabbits induced hyperlipidemia. Preparative density gradient centrifugation was applied for the simultaneous isolation of the major lipoprotein density classes i.e. VLDL, LDL, HDL forming discrete bands of lipoproteins in the preparative tubes. The cholesterol and protein values were determined in the fractions of lipoproteins. LDL was subsequently dialysed against physiological solution and sterilized and eliminated apolipoprotein fragments, aggregates by passing through a 0.22 micron filter. Radiolabelled of LDL with 99mTc was peformed using sodium dithionite as a reducing agent. Radiochemical purity and in vitro stability were controlled by paper chromatography. We have preparated modified lipoproteins with chemical agents and with radioactive irradiations. The oxidation of LDL was carried out by Cu++ ions and increased by gamma irradiation. The extent of oxidation was measured by spectrophotometry on he basis of absorbance values.

Investigation of LDL was taken for Schlieren analysis after adjusting the density of sera and under-layering by salt solution, in the spinning ultracentrifugation capillary band-forming cell. We obtained quantitative results by measuring the Schlieren areas between the sample curves and the reference baseline curve by computerised numerical and graphic technics. With this technique, we measured the concentration of human LDL and analysed rabbit LDL in induced hyperlipidemia. The radiolabelling of LDL was tested in atherosclerosis and in tumor cells on he bases of scintigraphic examinations in animal models and in vitro LDL uptake techniques. Rabbits were fed a diet containing cholesterol to develop hyperlipidemia and atheromatous aortic plaques. In mice were set human tumor cells to develop tumor tissues.

Rabbits fed a diet containing 2 % cholesterol for 60 days to develop hyperlipidemia and atheromatous aortic plaques. Two months after starting cholesterol feeding, the total cholesterol in blood serum increased approximately 20-30-fold in comparison to the basal cholesterol content of hypercholesterolemic rabbits, exceed 2000 mg/ dl cholesterol levels. Analyses for the principal lipid constituents were done on the whole serum and the isolated lipoprotein fractions. Serum cholesterol and triglycerides in serum and lipoproteins were determined by enzymatic assays using selective precipitation methods and spectrophotometric assays. Labelling efficiency were 85-90 % for human native LDL, for human oxidized LDL, for rabbit native IDL and for rabbit oxidized IDL.

Gamma scintillation camera scanning of the rabbits was peformed. Overnight fasted rabbits were injected in the marginal ear vein with 99m Tc-labelled human LDL (4-10 mCi, 0.5-1.5 mg protein.Gamma camera in vivo scintigraphy of live rabbits revealed visible signal corresponding to atherosclerotic plaques of aorta and carotid arteries.

During 3 months immunisation period we immunisated rabbits with 3-4 times with of human LDL.We obtained antibody against human LDL in rabbits blood samples with high concentration values.We have detected immunoprecipitation reaction in an extent of about 1000, 10000 fold of the dilution of sera.

We compared the LDL uptake of normal and pathological mononuclear cells with the analysis of the supernatant before and after incubation with LDL fraction with the analysis of the supernatant before and after incubation with LDL. The difference between the two curves indicates the LDL uptake by mononuclear cells in the in vitro model.It was detected LDL receptor or scavenger receptor uptake of lipoproteins in native state without labelled lipoproteins.The HC rabbits showed extensive presence of atherosclerotic lesions in the aorta and a detectable accumulation of 99m Tc LDL was observed in their aortas and in their carotid arteries. The atherosclerotic lesions of aorta arteries from cholesterol-fed rabbits, mainly in the aortic arch, accumulated more 99m Tc-LDL then aortas from control rabbits.

In nude mice developed human tumor cells were detected on the basis of 99mTc labelled LDL with Gamma camera. Image of a dog with spontaneous osteosarcoma in the right scapula was detected after injection of 600 MBq of native ^{99m}Tc-labelled human LDL. Agents widely used in clinical practice include: uptake and storage of radiolabelled cholesterol analogues via the low density lipoprotein (LDL) receptor and cholesterol ester storage pool. Radiolabelled lipoproteins which bind LDL receptors can be used for the scintigraphic detection of the atherosclerosis, the tumor cells and the imflammation processes.

7. CONCLUSIONS

The radiolabeling of LDL has been tested in atherosclerosis and in cancer. There are strong need for non-invasive techniques in directly imaging atherosclerotic lesions and tumour lesions for early detections. Radiolabelled LDL offer the promising approach to identify the local metabolic fate of these compounds and to study LDL accumulation in vascular tissue and tumour tissues, because LDL acts as a trapped ligand in vivo and should be a good tracer for the scintigraphic studies of atherosclerosis and cancer. We have developed rapid and reproducible ultra-centrifugation methods to obtain lipoprotein aliquots for radiolabeling and to evaluate lipoprotein labeling techniques. We have used special designed cells in the underlayering of lipoproteins and special written software for the computerized evaluation of the flotation of lipoproteins fractions. The Schlieren method provides a simple way to characterize lipoprotein distribution in the whole serum without resort to stepwise separated preparative centrifugation, which has many drawbacks. Combination of preparative and analytical ultracentrifugation methods can be used for the investigation of Lipoprotein (VLDL, IDL, LDL, HDL) aliquots to determine concentration values and the presentation of lipoprotein Schlieren data to gain insight of the profile allowing comparison of serum lipoprotein concentration. We think so that these simple ultracentrifugal methods may be used in many fields of the lipoprotein research. Hypercholesterolemia is an important risk factor for the development of the atherosclerotic process. Experiments were performed in order to permit direct comparison of the amounts of lipoprotein present in animal models in the cases of the experimental hyperlipidemia.

Atherosclerosis was induced in rabbits by a hypercholesterinemic diet. Two months after starting cholesterol feeding, the total cholesterol in blood serum increased approximately 30-40-fold in comparison to the basal cholesterol content of HC rabbits. Autopsies revealed the formation of atheromatous plaques on the inner aortic surfaces of rabbits in induced hyperlipiemia. Radiolabeled of LDL with 99mTc was peformed using sodium dithionite as a reducing agent. Radiochemical purity and in vitro stability were controlled by paper and sephadex gel chromatography. The radiolabeling efficiency was more than 90 %. Our preliminary results showed that gamma camera in vivo scintigraphy of rabbits revealed visible signal corresponding to atherosclerotic plaques of the aorta and the carotid arteries, which were confirmed by pahtological examinations. The radiolabelling of LDL has been approached in atherosclerosis. Gamma camera in vivo scintigraphy of live rabbits revealed visible signal corresponding to atherosclerotic plaques of aorta. Our results show that 99m Tc-LDL can be used to assess the organ distribution pattern of LDL in the rabbit, and to detect and localize areas of arterial atherosclerotic lesions as well. Gamma scintillation camera scanning of the mice and dogs were peformed to detection of tumor cells.

In the future we should like to investigate which radiolabeled lipoprotein fractions are better for early lesion detection of atherosclerosis. The in vivo imaging studies in rabbits in mice and in dogs suggest that noninvasive imaging of atherosclerotic plaques and tumor cells using radiolabeled LDL may be feasible in humans. Lipoproteins are endogenous particles that transport lipids through the blood to various cell types, where they are recognised and taken up via specific receptors. These particles are, therefore, excellent candidates for the targeted delivery of drugs to various tissues.

Lipoproteins from commercially available natural and synthetic lipids and serum-derived or recombinant apolipoproteins, which closely mimic the metabolic behaviour of their native counterparts have being to selectively deliver a wide range of lipophilic, amphiphilic, and polyanionic compounds to hepatocytes, plaques and tumour cells.

8. ACKNOWLEDGEMENTS

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